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Repeated Social Stress Induces Metaplasticity in Ventral Tegmental Area Dopaminergic Neurons

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Dedication

For the rats, who didn’t get a choice in this matter.

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Repeated Social Stress Induces Metaplasticity in Ventral Tegmental Area Dopaminergic Neurons

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Learned associations between environmental cues and drug reward are critical for reinforcing drug use and triggering relapse. Effective prevention and treatment of substance use disorders requires a detailed understanding of the synaptic plasticity mechanisms underlying these associations as well as their modulation by experience, environment, and genetic differences. Stressful experience increases susceptibility to addiction in humans and hastens the development of addiction-related behaviors in rodent models. The facilitating effects of stress persist beyond the duration of the stressful experience, suggesting that stress causes persistent cellular adaptations that promote drug-related learning processes. The data presented in this dissertation demonstrate that stress, via glucocorticoid receptor (GR) signaling, induces metaplasticity of N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic transmission in dopaminergic (DAergic) neurons of the ventral tegmental area (VTA). NMDAR-mediated transmission in these neurons is of particular importance as it is required for burst firing and subsequent phasic dopamine release, which is both

necessary and sufficient for learning of reward-predictive cues. Repeated exposure to social defeat stress induces a persistent, PKA-dependent increase in the sensitivity of inositol 1,4,5-triphosphate (IP_3) receptors. This sensitization of IP_3Rs augments metabotropic glutamate receptor (mGluR) -dependent calcium (Ca^{2+}) signaling, which then enhances induction of long-term-potentiation of NMDAR transmission (NMDAR-LTP). Repeated social defeat stress also enhanced acquisition of cocaine conditioned place preference (CPP), a form of associative learning driven by drug reward, and this effect required GR signaling as well. This dissertation provides the first demonstration of stress-induced metaplasticity in VTA DA neurons. These findings may illuminate one mechanism by which stress increases vulnerability to addiction, a chronic, relapsing disorder that is perpetuated by powerful memories of stimuli associated with drug reward.

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Chapter 1: Introduction

The brain is both the initiator and the target of the stress response, a set of physiological mechanisms that allow an animal to cope with insults to homeostasis (Selye, 1973). When the brain detects a threat, it engages a specific pattern of hormone release that alters metabolism, recruits inflammatory processes, and feeds back to the brain to alter neural function. Stress differentially alters the ability to acquire and consolidate distinct forms of memory (Kim and Yoon, 1998; Schmidt et al., 2013) and biases the use of certain memory systems at the expense of others (Kim et al., 2001; Schwabe and Wolf, 2012). In rodent models, activation of the stress response disrupts subsequent declarative learning and cognition, impairing spatial learning (Conrad et al., 1996; Luine, 2002; Luine et al., 1994), object recognition (Beck and Luine, 1999; Luine, 2002), and cognitive flexibility (Liston et al., 2006; Mizoguchi et al., 2000). In contrast to these deficits, prior stress enhances learning of Pavlovian cue-outcome associations. Learning may be driven by rewarding outcomes, assessed by conditioned place preference (Burke et al., 2011; Der-Avakian et al., 2007; Kreibich et al., 2009; Mathews et al., 2008), or aversive stimuli, assessed by fear conditioning (Conrad et al., 1999; Sandi et al., 2001; Suvrathan et al., 2013; Vogel et al.). The selective enhancement of Pavlovian learning may have arisen from evolutionary pressure to rapidly acquire information predicting food,

shelter, and predator threat under duress. While augmented Pavlovian reward learning may be adaptive in some situations, it also heightens susceptibility to addiction. Acquisition of cue-drug associations is a crucial early step in drug use, and powerful, enduring memories of drug-associated cues trigger craving and relapse as recreational use progresses to addiction (Berridge, 2012; Hyman et al., 2006; O'Brien et al., 1998; Piazza and Deroche-Gamonet).

Mesolimbic DAergic neurons, which originate in the VTA and project to the nucleus accumbens (NAc), are critically involved in learning cue-reward associations. DA neurons signal motivationally relevant information via brief, phasic changes in firing rate (Schultz, 2007). VTA DA neurons are excited by unexpected rewards, which trigger bursts of action potentials. These unconditioned DA reward signals are necessary for Pavlovian learning (Darvas et al.; Tsai et al., 2009). Over the course of learning, VTA DA neurons acquire a conditioned burst response to reward-predictive cues that is thought to encode the positive motivational valence of the cue and invigorate reward-seeking behavior (Berridge, 2012; Bromberg-Martin et al., 2010; Schultz, 1998). In the context of addiction, these learned phasic responses to drug-associated cues are likely triggers of relapse (Hyman et al., 2006). As NMDARs underlie the transition to bursting (Chergui et al., 1993; Grace and Bunney, 1984; Overton and Clark, 1997), long-term potentiation (LTP) of cue-driven NMDAR input to DA neurons may underlie conditioned bursting. Our lab has previously characterized NMDAR LTP induced by repeated pairing of cue-like synaptic stimulation with reward-like

bursting (Harnett et al., 2009). Additionally, repeated exposure to ethanol, psychostimulants, or an impoverished environment enhances NMDAR LTP by increasing the sensitivity of the LTP induction mechanism (Ahn et al., 2010; Bernier et al., 2011; Whitaker et al., 2013). This dissertation used the repeated social defeat model of stress to examine the effects of stress on metaplasticity of NMDAR transmission in VTA DA neurons and learning of Pavlovian reward associations.

THE STRESS RESPONSE

Definition and History

'Stress' is an elusive concept to define. Hans Selye, the endocrinologist who coined the term, famously quipped, "Everybody knows what stress is, and nobody knows what it is" (Selye, 1973). Scientific investigation of stress began in the 1930s, when Selye's research cataloguing the effects of various noxious stimuli (e.g. exposure to cold, injury, exercise to exhaustion, various drugs) on organ systems of the rat revealed that aversive experience universally recruited a common set of physiological responses (Neylan, 1998). Originally dubbed the 'General Adaptation Syndrome,' the response was divided into a rapid 'general alarm reaction,' followed by later symptoms of adrenal hypertrophy, gastric ulcers, fat and muscle loss, and lymphatic tissue atrophy (Neylan, 1998). In light of the emerging idea of homeostasis, first introduced by Walter Cannon (Cannon, 1932), Selye renamed this syndrome "stress," and refined its definition to "the

nonspecific response of the body to any demand made upon it" (Selye, 1973). The rapid and delayed responses that Selye observed were later shown to result from activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis, respectively.

Sensing Stressors

Stressors can be broadly broken into two classifications. Physical stressors (e.g. food deprivation, severe temperatures) actively disrupt homeostasis, and this visceral information is relayed to brainstem nuclei. Psychological stressors (e.g. predators, aggressive social partners) are perceived threats to well-being that must be recognized and evaluated by processing in sensory cortex, hippocampus, and amygdala. Both stressor types drive activation of parvocellular neuroendocrine cells (PNCs) within the periventricular nucleus of the hypothalamus (PVN) to provoke systemic stress responses.

Sympathetic-Adrenal Response

Commonly called the 'fight-or-flight response,' acute sympathetic nervous system activation by threatening stimuli was first described by Cannon in the late 1920s (Cannon, 1929). When a threat is sensed, the hypothalamus activates efferent sympathetic nerve fibers, which make cholinergic synapses in sympathetic ganglia to postganglionic fibers. These fibers provide noradrenergic

input to most organ systems in the body, resulting in bronchodilation, increased heart and breathing rate, and redirection of the blood supply from the organs to the skeletal muscles. Additionally, a direct preganglionic projection to chromaffin cells in the adrenal medulla stimulates secretion of epinephrine into the blood. Epinephrine exerts similar effects to norepinephrine, but additionally elevates blood glucose by stimulating glycogen breakdown in the liver. These effects occur within seconds to rapidly ready the body for the energy expenditure necessary to defeat or escape a threat. Epinephrine does not readily cross the blood-brain barrier and therefore has no effect on the central nervous system, but the visceral sensations of sympathetic activation are relayed back to the CNS, where they contribute to self-perception of stress and anxiety.

The Hypothalamic-Pituitary-Adrenal Axis Response

While the sympathetic-adrenal response readies the body for immediate conflict or escape, the HPA axis provides a slow, sustained response mediated by effectors that readily cross the blood-brain-barrier to influence neural activity (Ulrich-Lai and Herman, 2009). This biological pathway is triggered when PNCs of the PVN hypothalamus release corticotropin-releasing factor (CRF) into the local capillary blood supply of the anterior pituitary gland. There, CRF stimulates corticotropic endocrine cells, which secrete adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH triggers the adrenal cortex to secrete corticosteroid hormones, of which glucocorticoids (GCs) (primarily corticosterone in rodents)

are major effectors of the physiological response to stress. GC secretion begins within minutes of stressor onset and tends to peak approximately 30 minutes later. Glucocorticoids bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), nuclear receptors ubiquitously expressed throughout the body that act as transcription factors upon activation. MRs have high-affinity for corticosterone and are readily saturated by levels of achieved during normal circadian fluctuations, while lower-affinity GRs only begin to activate at the peak of circadian rhythm (Joels and de Kloet, 1994; Meijer and de Kloet, 1998; Meijer et al., 1998). GR activation has diverse, tissue-specific effects including reducing inflammation, promoting glucose conservation, and breaking down fats. Glucocorticoids readily cross the blood-brain barrier to regulate gene expression in neurons. There is also evidence of rapid, non-genomic GC effects that appear to be mediated by membrane-bound, G-protein coupled GRs (Tasker et al., 2005). Rapid GC responses are typically excitatory and increase spiking, with the exception of rapid feedback inhibition on PVN PNCs (Tasker et al., 2005). The HPA stress response is self-limiting, shutting itself off by a set of glucocorticoid-mediated negative feedback mechanisms including the aforementioned nongenomic fast feedback on PVN PNCs as well as feedback inhibition mediated by GRs in the hippocampus and, to a lesser degree, the prefrontal cortex (Herman et al., 2012). Dysregulation of these feedback mechanisms have been implicated in the etiology of depression and post-traumatic stress disorder (Herman et al., 2012).

Adverse experience reliably evokes GC secretion in humans and animal models, and elevated GC levels correlate with subjective reports of 'stressful' emotional states in humans (Wolf et al., 2001). For these reasons, circulating GC levels are often used as an index of the intensity of the stress response, but there are caveats to this practice. First, GC levels are also elevated by pleasurable, rewarding stimuli (Bronson and Desjardins, 1982; Mantsch et al., 2000; Phoenix et al., 1977; Rosmond et al., 2000), so GC secretion cannot be the only requirement for classifying a stimulus as a stressor. Second, a given stressor can produce very different neurophysiological and behavioral effects if it is escapable *vs.* inescapable (Drugan et al., 1997), but GC responses typically do not differ with escapability (Maier et al., 1986; Shors et al., 1989).

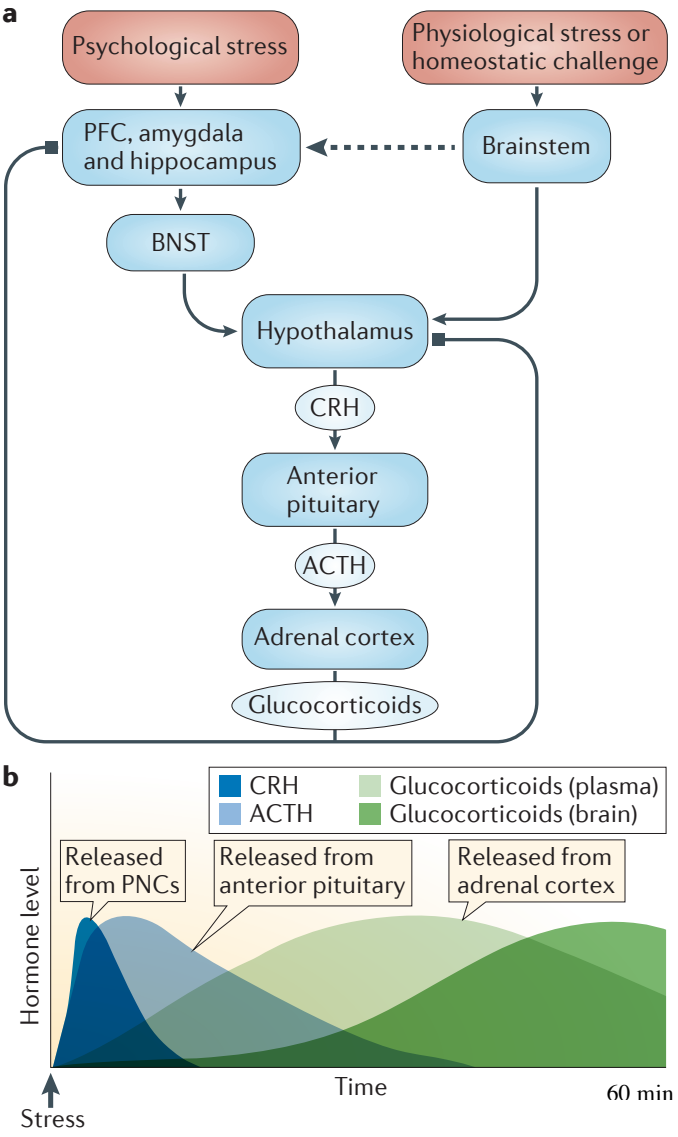


Figure 1. Hypothalamic-pituitary-adrenal axis

(**A**) Schematic of the brain and endocrine structures involved in initiation and execution of the hypothalamic-pituitary-adrenal stress response by physical or psychosocial stress. (**B**) Time course of HPA hormone release following exposure to stress. Plasma glucocorticoids peak at approximately 30 min and return to basal levels by approximately 2 h. CRF, corticotropin releasing factor; ACTH, adrenocorticotropic hormone; PFC, prefrontal cortex; BNST bed nucleus of stria terminalis, Adapted from (Bains et al., 2015).

Within-brain Stress Responses: CRF and NE

While the brain coordinates bodily responses to stress with the sympathetic-adrenal and HPA pathways, it also coordinates physiological and behavioral responses via within-brain mechanisms. CRF is released from hypothalamic PNCs as well as from neurons within extrahypothalamic sites, including the central amygdala, bed nucleus of the stria terminalis (BNST), locus coeruleus (LC) and the nucleus of the solitary tract (Carrasco and Van de Kar, 2003). There are two families of receptors activated by CRF, CRFR1 and CRFR2, and a CRF-binding protein regulates free CRF levels. CRFR1 is expressed widely, including in the cortex, anterior pituitary, amygdala, midbrain, cerebellum, and hippocampus. CRFR2 expression is localized to subcortical structures, including the hypothalamus, midbrain, amygdala, and hippocampus. Both CRF receptors are GPCRs, but G-protein coupling of each type varies by tissue. Behavioral effects of extrahypothalamic CRF are dependent upon arousal state, as central CRF delivery evokes increased locomotion and grooming from rats in a familiar environment but potentiates defensive and anxiety-like behaviors in a novel environment (Koob and Heinrichs, 1999; Sarnyai et al., 2001). Interestingly, CRF release from the amygdala is potentiated by GR activation, suggesting that there is a feed-forward loop promoting anxiety-like behaviors nested within the greater negative feedback scheme of the HPA axis (Cook, 2002) (Cook, 2004; Herman et al., 2012).

Stressful stimuli strongly activate noradrenergic neurons in the locus coeruleus and nucleus of the solitary tract. The locus coeruleus is reciprocally connected with hypothalamic CRF neurons and assists in the activation of the HPA axis, but also supplies NE input to the midbrain, cortex, and cerebellum, where its activation promotes attention and arousal. NE activates the G-protein coupled α and β adrenergic receptors. α_1Rs couple to G_q, α_2Rs are typically autoreceptors coupled to G_i, and β_1Rs and β_2Rs couple to G_s.

REGIONAL DIFFERENCES IN STRESS EFFECTS ON PLASTICITY & BEHAVIOR

Stress can exert dramatically different effects on plasticity and behavior depending upon (1) relative timing of the stressor and the plasticity/behavior, (2) cumulative stressful experience prior to plasticity/behavior, and (3) brain region in which plasticity/behavior is examined. In terms of timing, stress typically improves memory consolidation for events occurring simultaneously with the stressor (McGaugh and Roozendaal, 2002); however, this effect is not relevant to this study's research question and is therefore not reviewed in detail.

Hippocampus

Acute or chronic stress impairs acquisition of hippocampal-dependent tasks (Diamond et al., 1996; Kim et al., 2001; Luine et al., 1994). These deficits are accompanied by a shift in the balance of LTP and LTD induction. Single

stress exposures impair subsequent LTP induction in CA1, CA3, and dentate gyrus (Chen et al.; Foy et al., 1987; MacDougall and Howland; Shors and Dryver, 1994) while enhancing induction of LTD in CA1 (Kim et al., 1996; Xu et al., 1997). These effects can be mimicked by treatment with stress levels of corticosterone (Diamond et al., 1992; Kerr et al., 1994; Pavlides et al., 1993). Prolonged stress exposure similarly impairs LTP (Pavlides et al., 2002) and additionally produces structural plasticity in the form of dendritic atrophy and spine loss in CA3 (Chen et al., 2008; Magarinos and McEwen, 1995).

Prefrontal Cortex

Studies have found a similar pattern of stress-induced behavioral and plasticity deficits in the prefrontal cortex as those described in the hippocampus. Acute stress impairs performance of PFC-dependent tasks; however, young animals are resilient to this effect (Liston et al., 2006; Mizoguchi et al., 2000; Yuen et al., 2009). Chronic stress impairs PFC function regardless of age (Bondi et al., 2007; Yuen et al.). LTP is impaired in some PFC subdivisions following acute or chronic stress (Cerqueira et al., 2007; Goldwater et al., 2009; Maroun and Richter-Levin, 2003; Rocher et al., 2004). Prolonged stress exposure also induce spine loss and dendritic retraction in the PFC (Cook and Wellman, 2004; Liston et al., 2006; Radley et al., 2008; Radley et al., 2004).

Amygdala

The findings of stress-mediated changes in the amygdala contrast sharply with those observed in the hippocampus and PFC. Acute or chronic stress enhances subsequent amygdala-dependent learning (Conrad et al., 1999; Cordero et al., 2003; Shors and Mathew, 1998; Vogel et al., 2015; Vyas et al., 2004). Chronic stress produces dendritic hypertrophy in BLA projection neurons and induces spine formation (Mitra et al., 2005; Vyas et al., 2002; Vyas et al., 2004). Interestingly, even a single stress experience results in a slow-onset increase in spines (Mitra et al., 2005). The role of corticosterone in these changes is difficult to interpret, as corticosterone treatment can reproduce some forms of stress-induced structural plasticity but, intriguingly, appears protect against others (Mitra and Sapolsky, 2008; Rao et al.). Acute or repeated stress enhances LTP induction in the basolateral amygdala (Manzanares et al., 2005; Sarabdjitsingh et al., 2012; Suvrathan et al., 2013; Vouimba et al., 2006; Vouimba et al., 2004). Several factors contribute to this effect, including increased excitability due to disinhibition and the addition of silent synapses with greater capacity for LTP.

STRESS-INDUCED METAPLASTICITY

The concept of metaplasticity, the “plasticity of plasticity,” emerged from *in vitro* studies of LTP induction, when it was discovered that a neuron’s history of synaptic activity could influence the subsequent induction of LTP (Huang et al.,

1992) and, later, LTD (Mockett et al., 2002). Metaplasticity was originally defined as an activity-dependent change in the ability of synapses to undergo plasticity that persists after the priming activity has ended (Abraham, 2008). The pattern of observed changes in LTP/LTD induction were found to fit well with the Bienenstock-Cooper-Munro (BCM) computational model of synaptic plasticity (Abraham, 2008), which was first developed to model experience-dependent plasticity during the critical period in visual cortex (Bienenstock et al., 1982). In this adaptation of the BCM model, the magnitude and direction (LTP vs. LTD) of synaptic plasticity are dependent upon postsynaptic activity (and likely Ca^{2+} concentration) during induction, and the modification threshold, θ_m, describes the crossover point from LTD to LTP. Prior synaptic activity induces metaplasticity by shifting θ_m, presumably by temporary state changes in Ca^{2+}-dependent kinases and/or phosphatases. The effect of acute stress or glucocorticoid treatment on subsequent induction of LTP and LTD in hippocampal CA1 neurons also resembles a shift in θ_m. (Kim and Yoon, 1998). GR-mediated increases in L-type Ca^{2+} channel expression may underlie this effect (Coussens et al., 1997). These findings broadened the definition of metaplasticity to include longer-lasting, synaptic activity-independent events such as hormone secretion as 'priming activity' (Abraham, 2008).

Although the molecular mechanisms involved are not well understood, amygdala activation during stress also appears to modulate LTP in the hippocampus and PFC in a way that resembles metaplasticity. Inactivating the

amygdala during an acute stressor prevents stress-induced impairment of LTP in CA1, and this effect is independent of glucocorticoid signaling (Kim et al., 2005; Kim et al., 2001). Similarly, priming stimulation of the amygdala mimics the effect of prior stress, impairing LTP induction in the mPFC (Richter-Levin and Maroun, 2010).

STRESS, ADDICTION RISK, AND MEMORY

The above findings demonstrating dramatic effects of stress on learning and synaptic plasticity may provide insight as to how stress influences addiction risk. Humans with a history of stressful or traumatic experience are more prone to develop substance use disorders (Sinha, 2008; Turner and Lloyd, 2003). Similarly, stress promotes development of addiction-like behaviors in animal models using various classes of drug. Prior stress enhances conditioned place preference, a form of Pavlovian reward learning (Burke et al., 2011; Der-Avakian et al., 2007; Kreibich et al., 2009; Mathews et al., 2008). Animals with a history of stressful experience display increased drug self-administration (Becker et al., 2011; Boyson et al., 2014; Goeders and Guerin, 1994; Hadaway et al., 1979; Han et al., 2015; Piazza et al., 1990; Shaham and Stewart, 1994). Stress also sensitizes subsequent drug-evoked DA release in the NAc, although this effect has only been tested with psychostimulants (Boyson et al., 2014; Garcia-Keller et al., 2013; Han et al., 2015; Pacchioni et al., 2006). This effect is mediated by CRFRs, as pretreatment with a CRFR1 antagonist before stress dampens cross-

sensitization of DA release, and CRFR2 pretreatment blocks it entirely (Boyson et al., 2014).

The development of addiction is thought to be a multi-step process, starting with recreational use for hedonic effects, progressing with escalating usage despite negative consequences, and culminating in chronic cycles of abstinence and relapse (Piazza and Deroche-Gamonet, 2013). Pathological reward learning driven by drugs is thought to play a major role in this process. In the early stages of drug use, drug-related cues are learned to have extremely high motivational value. These powerful, enduring associative cue memories trigger craving and relapse as recreational use progresses to addiction (Berridge, 2012; Hyman et al., 2006; O'Brien et al., 1998). Stress may therefore promote addiction by modulating the synaptic plasticity mechanisms underlying acquisition and extinction of these drug-associated memories.

ROLES OF DA: REWARD, REINFORCEMENT, AND MOTIVATION

Food, water, sex, and other stimuli that promote survival and reproduction are 'rewarding' – that is, they evoke pleasurable sensations, elicit approach and consummatory behaviors, and serve as behavioral reinforcers. DA neurons in the ventral midbrain – the substantia nigra pars compacta and VTA - are critically involved in reward processing and the acquisition and expression of motivated behaviors (Bromberg-Martin et al., 2010; Wise, 2004). The first evidence of a neural system for reward and reinforcement came from studies of electrical

stimulation of the lateral hypothalamus and medial forebrain bundle by Olds and Milner in the early 1950s. These electrode placements stimulate the mesolimbic and mesocortical dopamine systems, which consist of DAergic projections originating in the VTA and terminating in the NAc and PFC, respectively. Animals exhibit a learned preference for places in which they received stimulation and will perform behavioral tasks in order to receive stimulation (Olds and Milner, 1954; Olds, 1958). Humans will voluntarily perform intracranial self-stimulation, which they describe as a pleasurable experience (Bishop et al., 1963). These early studies established that direct activation of certain brain regions could support both Pavlovian and operant reward-based conditioning. In Pavlovian reward paradigms such as conditioned place preference (CPP) or Pavlovian conditioned approach (PCA), a learned association between a previously neutral cue and rewarding outcome provokes a conditioned appetitive response to the cue. In operant reward paradigms, such as lever-pressing for food pellets, drug infusions or intracranial stimulation, a learned contingency between a neutral cue, an action response to that cue, and a rewarding outcome provokes conditioned action performance in response to cue presentation.

Studies that pharmacologically or genetically prevented mesolimbic DA signaling confirmed its necessity for learning the motivational value of reward-associated cues and performance of motivated behavior. DA signaling is required for acquisition of Pavlovian conditioned responses (Darvas et al., 2014; Flagel et al., 2011; Spyraki et al., 1982) and operant responding for rewards (Wise and

Schwartz, 1981). Animals that have successfully acquired operant behavior show progressive reductions in responding under DA blockade (Fouriezos and Wise, 1976; Wise et al., 1978). A study using the operant drug self-administration paradigm revealed that DA is involved in the execution of learned operant responses (Phillips et al., 2003). The authors observed DA release time-locked to the animal's operant response, and electrically evoked DA release was sufficient to drive operant responses. There is also substantial evidence that mesolimbic DA is necessary to support expenditure of effort in goal-directed behavior. DA antagonists reduce the breakpoint in progressive ratio schedules of reinforcement (Hamill et al., 1999), and animals with impaired mesolimbic DA signaling choose to perform low-effort/low reward responses over high-effort/high reward options (Cousins et al., 1993). Together, these studies established a central role for mesolimbic DA in learning of cue-reward and cue-action-reward relationships as well as motivating previously acquired behavioral responses to obtain reward.

VTA DA Circuitry

The VTA, located in the ventral midbrain, is comprised of approximately 60-65% DAergic neurons, 35% GABAergic neurons, and 2-3% glutamatergic neurons (Sesack and Grace, 2010). DA neurons are tonically active, with basal firing frequency set by intrinsic pacemaker conductances and modulated by synaptic input. Pacemaking is driven by interplay of background Na^+ leak

conductance and 'persistent' voltage-gated Na^+ conductance, and intrinsic HCN and SK conductances contribute to the firing rate (Khaliq and Bean, 2010). DA neurons respond to motivationally relevant stimuli with phasic increases (bursts) or decreases (pauses) in firing rate. NMDAR activation is both necessary and sufficient to evoke phasic bursting, although a more complex interplay with GABAergic input may regulate bursting activity (Chergui et al., 1993; Lobb et al., 2010; Lobb et al., 2011; Overton and Clark, 1997; Zweifel et al., 2009).

VTA DA neurons primarily project to the NAc and PFC, with lesser projections to the amygdala and BNST. These projections are mostly non-overlapping, and recent studies have found significant differences in electrophysiological characteristics among VTA neurons of different projection targets (Lammel et al., 2014). PFC-projecting DA neurons cluster in the medial VTA and have higher tonic firing rates, higher frequency of bursting, high basal AMPA: NMDA, and low expression of DAT, D_2 autoreceptors, and HCN. DA neurons projecting to the amygdala, NAc core, and medial NAc shell are also localized to the medial VTA and are electrophysiologically similar to the PFC-projecting population. Lateral NAc shell-projecting DA neurons are found in the lateral VTA and exhibit slower tonic firing rates, low basal AMPA: NMDA, and large HCN currents (Lammel et al., 2011). The NAc shell-projecting population is thought to be especially important for addiction-related learning, as drug-evoked DA release is highest in the shell (Pontieri et al., 1995) and lesions of DA terminals in the shell impairs acquisition and expression of conditioned place

preference (Sellings and Clarke, 2003). The shell may also be a site of particular importance for stress-reward interactions, as stress-evoked DA release is higher and drug-evoked DA release is modulated by corticosterone in this region (Barrot et al., 2000; Kalivas and Duffy, 1995).

Phasic DA neuron burst responses are driven by glutamatergic input (Grace and Bunney, 1984), and there are several excitatory projections to the VTA of approximately equal density (Watabe-Uchida et al., 2012). Mesoprefrontal DA neurons receive excitatory input from the PFC and habenula, while lateral shell mesoaccumbens DA neurons receive glutamatergic and cholinergic input from the laterodorsal tegmental nucleus (Carr and Sesack, 2000; Lammel et al., 2012). Other glutamatergic sources include projections from the BNST (Georges and Aston-Jones, 2002) and the lateral hypothalamus, which co-release peptide transmitters such as orexin and neurotensin (Kempadoo et al., 2013). DA neurons are tonically inhibited by spontaneously active local GABAergic neurons. Direct GABAergic projections from the NAc core and shell to VTA DA neurons have recently been confirmed (Watabe-Uchida et al., 2012), although the NAc also supplies inhibitory input to VTA GABA neurons and therefore can disinhibit the DAergic population. The rostromedial tegmental nucleus, which is downstream of the habenula, preferentially inhibits lateral shell-projecting mesoaccumbens population. Other sources of GABAergic input include the pallidum and central amygdala. Neuromodulatory pathways include a

dense serotonergic input from the dorsal raphe and a sparse noradrenergic input from the locus coeruleus (Watabe-Uchida et al., 2012).

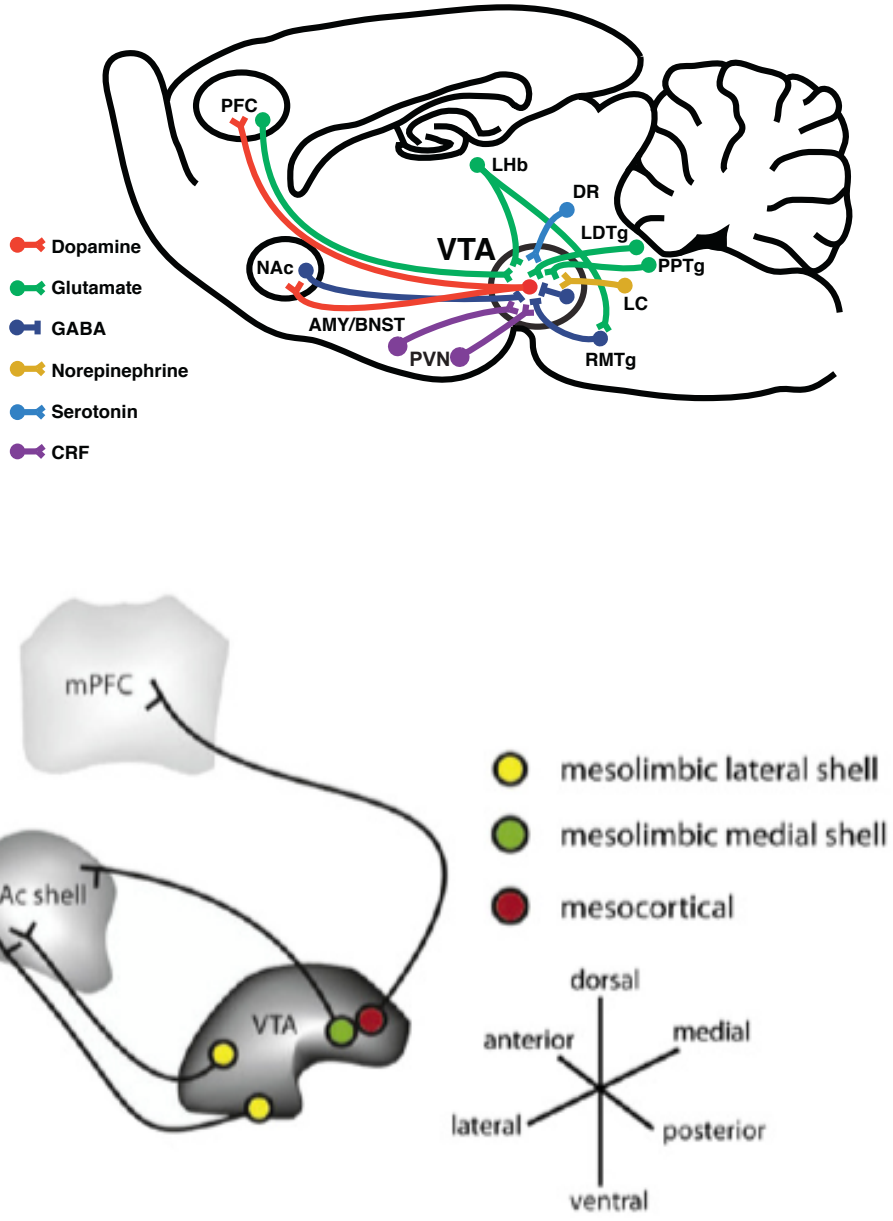


Figure 2. Mesocorticolimbic dopamine circuitry

Simplified schematic diagram highlighting the major inputs (above) and outputs (below) of the VTA. NAc, nucleus accumbens; PFC, prefrontal cortex; LHb, lateral habenula; DR, dorsal raphe nucleus; LDTg, laterodorsal tegmental nucleus; PPTg, pedunculopontine tegmental nucleus; LC, locus coeruleus; RMTg, rostromedial tegmental nucleus; LH, lateral hypothalamus; AMY, amygdala; BNST, bed nucleus of the stria terminalis. Adapted from Bernier 2011 and (Lammel et al., 2011).

DA Responses to Rewarding, Aversive, and Stressful Stimuli

Recordings of *in vivo* DA neuron activity in awake, behaving animals have informed current theories of dopamine function in motivated behavior. Wolfram Schultz's seminal studies of DA neuron activity in awake macaques found that rewards phasically excite DA neurons. Furthermore, Pavlovian conditioning induces a shift in the phasic excitation from the primary reward to the reward-predictive cue, and reward omission evokes phasic inhibition of firing. This activity pattern resembles a reward prediction error (RPE), i.e. it encodes the difference between expected reward and actual outcome (Schultz, 1998). RPE-like DA signals have also been observed in rodents (Cohen et al., 2012; Pan et al., 2005).

Studies of DA responses to aversive stimuli have revealed additional non-RPE DA signals. Aversive stimuli can either excite or inhibit DA neurons (Brischoux et al., 2009; Ungless et al., 2004), and early reports of excitation by noxious stimuli undermined the prevailing theory of DA reward prediction errors. Recent exhaustive characterization of DA neuron responses to noxious stimuli concluded that DA neurons display either of two response patterns (Matsumoto and Hikosaka, 2009), and these patterns exist along an anatomical gradient (Bromberg-Martin et al., 2010). 'Value-coding' neurons are phasically activated by rewards and inhibited by aversive stimuli, a pattern consistent with a role in prediction error signaling. These cells are most prevalent in ventromedial

midbrain. In contrast, 'salience-coding' neurons are phasically activated by both rewarding and noxious stimuli as well as cues predicting either type of stimulus. Salience-coding cells dominate the dorsolateral midbrain but are also found interspersed in extreme ventromedial midbrain. Salience-coding neurons relay the presence and magnitude of motivationally salient stimuli, regardless of their rewarding or aversive nature, to the dorsolateral striatum, dorsolateral PFC, and NAc core to support orienting responses and general motivation. Value-coding neurons relay both magnitude and valence of motivationally relevant stimuli to the NAc shell, dorsal striatum, and ventromedial PFC, potentially supporting learning of the motivational valence of cues and approach or escape behavior. Differential projection patterns of the lateral habenula, which is also involved in phasic inhibition during negative prediction error (Matsumoto and Hikosaka, 2007), are implicated in the disparate responses to aversive stimuli in the VTA. Lateral habenula stimulation directly excites PFC-projecting DA neurons, but indirectly inhibits the lateral NAc shell-projecting population via the RMTg (Lammel et al., 2012). However, an aversive stimulus (painful formalin injection to the paw) increased the ratio of AMPARs to NMDARs in lateral NAc shell-projecting neurons (Lammel et al., 2011). Given their innervation by the RMTg, the neurons are expected to be inhibited by aversive stimuli; yet this form of AMPAR plasticity is NMDAR-dependent and typically observed after manipulations that activate DA neurons (Stuber et al., 2008; Ungless et al., 2001). As mentioned previously, the lateral shell-projecting population is heavily

implicated in responses to drugs of abuse, so this discrepancy warrants further study to better clarify the effect of aversive or stressful stimuli on these neurons.

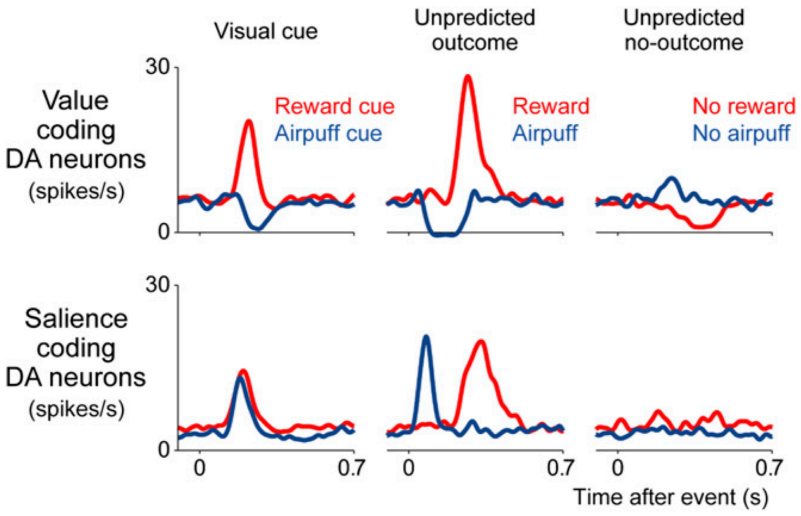
Microdialysis and voltammetry studies have established that stress increases DA concentration in most DAergic projection areas. VTA DA neurons exhibit increased bursting during stressful experience (Anstrom et al., 2009; Anstrom and Woodward, 2005). As stress induces CRF release into the VTA (Wang et al., 2005), bursting during stress may be augmented by transient, global CRFR2-mediated enhancement of NMDARs (Ungless et al., 2003), as CRFR2 blockade attenuates stress-induced DA release in the NAc (Holly et al., 2015). There is also evidence for stress-induced changes in GR-mediated afferent control by D1-expressing medium spiny neurons in the NAc, as selective deletion of GRs in these neurons attenuates stress-induced DA release (Barik et al., 2013). The simplest explanation for these findings is that stressors are often aversive, therefore phasic excitation of salience-coding DA neurons underlies stress-induced DA release. However, the anatomical patterns of stress-induced DA elevation are not fully consistent with the presumed projection targets of salience-coding neurons. Stress-evoked DA release in the PFC, dorsal striatum, and NAc core is unsurprising given these regions' targeting by the salience-coding population (Abercrombie et al., 1989; Anstrom et al., 2009; Butts et al., 2011; Holly et al., 2015). However, it has also been repeatedly shown that stress also induces substantial DA release in the NAc shell, which is primarily innervated by value-coding DA populations (Barik et al., 2013; Holly et al., 2015;

Kalivas and Duffy, 1995). DA release persists with repeated exposures to the stressor and therefore cannot be explained as a 'novelty' or 'alerting' DA response. To reconcile these findings, further must clarify how patterns of DA activation vs. inhibition may differ between simple aversive stimuli and complex stress experiences and identify which input(s) mediate the excitation induced by stressful and/or aversive stimuli in distinct VTA subcircuits.

Prolonged, unrelieved exposure to stress, which has been implicated in the development of depression-like behaviors, can induce altered patterns of activity in VTA DA neurons, in some cases persisting for several days. Studies have yielded conflicting results, possibly due to differences in the duration or severity of the stressor used. Chronic food deprivation enhances bursting (Branch et al., 2013), and chronic social defeat increases both tonic and burst firing, although effects may be limited to a subset of stress-susceptible animals (Barik et al., 2013; Cao et al., 2010; Razzoli et al., 2011). In contrast, chronic mild stress decreased tonic firing and bursting in putative DAergic neurons (Tye et al., 2013). Depression-like behaviors such as reduced sucrose preference and social avoidance may emerge as a consequence of chronic stress (Krishnan et al., 2011); however the relationship of tonic and phasic DA to these depression-like behaviors is likely extremely complex. Depression-like behaviors emerged regardless of whether stress induced hyperactivity or hypoactivity of DA neurons (Cao et al., 2010; Tye et al., 2013), and phasic DA neuron stimulation could either induce or relieve depression-like behaviors (Chaudhury et al., 2013; Tye et

al., 2013). Chronic stress also decreases population activity in the VTA, which may affect tonic DA levels and the net intensity of phasic DA signals in projection targets (Moore et al., 2001; Valenti et al., 2012). Given the high comorbidity of depression and substance use disorders, further studies clarifying the relationship between stress, DA, and depression will likely yield insights to addiction as well.

A



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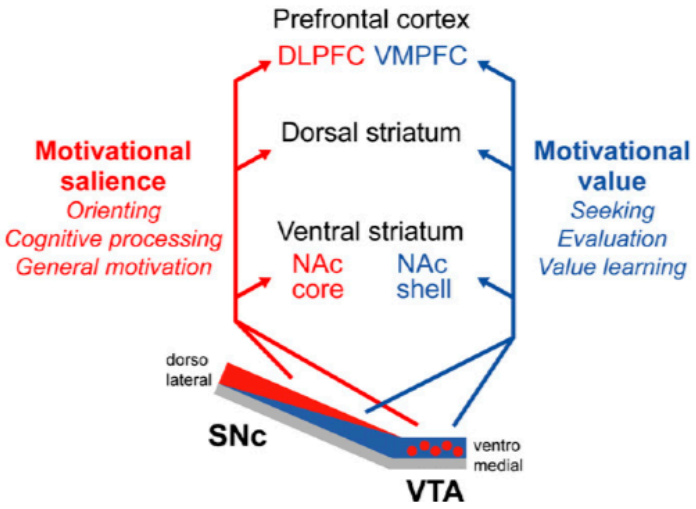

Figure 3. Value and salience coding in DA neurons.

(**A**) Responses of value-coding (above) and salience-coding (below) VTA DA neurons to rewarding and aversive outcomes and their predictive cues. Stimuli (visual cue, water reward, or aversive airpuff to the face) are delivered at t=0. Value-coding neurons are phasically excited by unexpected rewards or reward-predictive cues and phasically inhibited by unexpected aversive stimuli or aversive stimuli-predictive cues. Salience-coding neurons are phasically excited by both unexpected rewards and unexpected aversive stimuli as well as by cues predicting both rewarding and aversive outcome. (**B**) Anatomical location and putative projection targets of value-coding and salience-coding neuronal populations. Adapted from (Bromberg-Martin et al., 2010)

DA Effects in Projection Targets

The prevailing theories of DA's role in reward learning and addiction assume that phasic DA release in the NAc enables synaptic plasticity at corticostriatal synapses, and *in vitro* studies have suggested that synaptic plasticity of these synapses is bidirectionally modulated by DA concentration (Reynolds and Wickens, 2002). Striatal medium spiny neurons are segregated into D1 and D2 DA receptor-expressing populations that participate in distinct output circuits: the 'direct pathway' permits action via thalamic disinhibition, and the 'indirect pathway' suppresses action. The high-affinity D1 receptors are preferentially activated by concentrations of DA achieved with phasic bursting, and while D2 receptors were previously thought to be saturated by tonic levels of DA and only responsive to DA pauses, recent studies have demonstrated rapid activation of D2 by phasic DA (Marcott et al., 2014; Schultz et al., 1997). The downstream consequences of DA neuronal activity may be even more complex than previously thought in light of studies demonstrating co-release of glutamate or GABA from midbrain DA neurons (Tecuapetla et al., 2010; Tritsch et al., 2012). Much more research is needed to clarify the conditions in which fast transmission is used and the interaction that co-released small molecule transmitters may have with DA in the regulation of corticostriatal plasticity.

THEORIES OF DA FUNCTION IN REWARD LEARNING AND ADDICTION

There are several theories seeking to explain the development of addiction from the acute and repeated effects of drug use, and all are rooted in the idea that drugs of abuse 'hijack' natural reward mechanisms. Drugs acutely activate the mesocorticolimbic DA circuit, and chronic drug use induces lasting neuroadaptations that alter the circuit's function. The section below will summarize current theories of DA function in associative learning, motivation, and executive function. Each theory of DA function has generated putative mechanisms by which drug-induced changes in DA signaling may produce the hallmarks of addiction, compulsive drug use despite negative consequences and relapse after prolonged abstinence.

Drugs of Abuse

It has been known for some time that all abused drugs have the ability to acutely release DA in the NAc, dorsal striatum, and PFC, often to a greater degree than natural rewards (Wise, 1998). Drugs may elevate DA by increasing neuronal firing frequency, promoting vesicular release, or prolonging the action of released DA by inhibiting uptake. Psychostimulants such as cocaine and amphetamine prevent uptake by competitively inhibiting DAT, and amphetamine further releases DA from terminals by driving DA from vesicles into the cytoplasm, then out of the cell via reverse DAT transport. Opioids such as heroin and morphine inhibit VTA GABAergic neurons, thereby increasing DA neuronal

firing by disinhibition. Nicotine activates DA neurons via excitatory nicotinic receptors. Ethanol increases DA firing by a number of mechanisms including enhancing presynaptic release of acetylcholine and glutamate to DA neurons and inhibition of I_H.

Covey *et al.* recently put forth a compelling argument that the critical drug mechanism for addiction is not general elevation of DA levels, but specifically the production of DA transients via phasic burst responses (Covey et al., 2014). Burst-evoked DA transients are both necessary (Zweifel et al., 2009) and sufficient (Tsai et al., 2009; Witten et al., 2011) to drive reward learning, thus drugs must create DA transients in order to drive learning of drug-associated cues. Although classical mechanisms of drug action could increase the amplitude or duration of existing transients, only bursting can create new transients necessary for conditioning. New findings indicate that psychostimulants, previously thought to impair bursting via D2-mediated autoinhibition, do in fact induce phasic DA bursting (Daberkow et al.; Koulchitsky et al.). Taking into account these newfound psychostimulant actions, all classes of abused drugs generate DA burst responses, and these phasic DA signals are likely critical for learning Pavlovian cue-drug associations.

Reward Prediction Error

As discussed previously, phasic DA responses by value-coding neurons during Pavlovian reward learning bear a striking resemblance to the prediction

error term of the temporal difference (TD) class of model for reinforcement learning in machines (Glimcher, 2011; Schultz et al., 1997; Sutton, 1988). TD learning models are designed to predict the total value of future rewards. These models are continuous; reward predictions are constantly made, then updated by RPEs, which act as teaching signals. Unexpected reward delivery or omission and cues containing novel information about the probability of reward delivery each generate RPEs.

$$\text{RPE: Prediction Error (t)} = R_t + V(S_{t)} - V(S_{t-1})$$

Here, prediction errors are calculated at every moment in time, designated as states (S). R_t represents actual reward value at the given moment, $V(S_{t)}$ is expected value at the given moment which can be changed by informative cues, and $V(S_{t-1})$ is expected value one moment prior (Keiflin and Janak, 2015).

$$\text{Updating Rule: } V(S_{t-1})\text{new} = V(S_{t-1})\text{old} + \eta\text{Prediction Error(t)}$$

Here, updating is driven by RPEs and controlled by the learning rate η. Learning rates range from 0-1 and determine the 'reactivity' of the system to the error such that low learning rates will update the reward expectation by a small proportion of the error while high learning rates make more extreme adjustments. Models frequently also incorporate temporal discounting, such that in computing total

reward expectation, a given reward's value is reduced the farther in the future it is expected to occur. TD reinforcement learning models extend the basic framework to incorporate action-outcome contingencies and seek to maximize reward receipt by selecting actions with the highest reward outcome (Redish, 2004). The basal ganglia are thought to function as an actor-critic TD reinforcement model. In actor-critic models, the action choice policy ("actor") and the value function ("critic") are represented in separate nodes that are both updated by the error signal. The actor receives cue information from the environment and maps the cue to a behavior. The critic maintains a model-free, common-currency value prediction of the cue, and the TD module calculates the prediction error and feeds the error separately to the actor and the critic. In this scheme cue-action associations and cue-value associations are stored separately, but learning in both is driven by reward prediction error (McDannald et al., 2012). The dorsolateral striatum is thought to function as the actor, the ventral striatum as the critic, and the VTA as the error signal. It is unclear whether DA neurons compute error themselves, receive error information computed upstream, or some combination of the two. Recent evidence suggests that the VTA also receives model-based information about specific features of reward from the orbitofrontal cortex, suggesting a more complex mechanism in the basal ganglia than a purely model-free system like TDRL (McDannald et al., 2012; Takahashi et al., 2011).

These models have provided attractive explanations for the role of DA RPE-like signals in reward learning, but could not be rigorously tested until recent advances in optogenetic techniques allowed investigators to exclusively manipulate DA neuronal firing with temporal precision. One such study makes strong case for DA RPEs as drivers of reward learning (Steinberg et al., 2013). TD models (and other error-driven learning frameworks) predict a phenomenon called blocking. If a cue 'A' is learned to predict a reward and the reward is given as expected, the RPE is zero. If a novel cue 'B' is presented simultaneously with A and reward occurs as expected, the lack of RPE 'blocks' learning about B. An artificial RPE evoked by phasic optogenetic stimulation of DA neurons was able to unblock a previously blocked cue, further cementing DA's role as an error signal that drives learning.

TD-based learning models are designed to continually adjust an expected reward value until the expectation matches the outcome, i.e. the reward prediction error is zero. When natural rewards are predicted, it is likely that inhibition timed to expected reward delivery nullifies reward-induced excitation to achieve this zero error signal (Aggarwal et al., 2012). In the context of addiction, the abnormally large and prolonged DA elevation elicited by drugs creates a standing positive prediction error, leading to persistent overvaluation of drug-associated cues and drug-seeking actions relative to natural rewards. As a user repeatedly takes drugs, this ever-growing valuation leads the user to choose

drug-seeking actions over pursuit of natural rewards, and in spite of negative consequences (Montague et al., 2004; Redish, 2004).

Incentive Salience

In 1993, Robinson & Berridge introduced the incentive-sensitization theory, which seeks to explain the persistence of cue-induced craving and compulsive drug seeking after long periods of abstinence. In this scheme, the mesolimbic DA system assigns motivational importance or 'incentive salience' to reward-associated cues. These cues become wanted, invigorate ongoing actions, and serve as reinforcers. For example, a subset of animals that have learned a cue-reward association exhibit sign-tracking - they will orient to, approach, and even interact with the cue. The cue will increase the rate of operant responding for a different reward, an effect known as Pavlovian-instrumental transfer. Animals will perform operant tasks to access the cue, an effect known as conditioned reinforcement. 'Wanting' is distinguished from hedonic 'liking', as animals that cannot synthesize DA still display hedonic reactions to sweet tastes but cannot seek out or learn about the reward.

Repeated drug use is hypothesized to induce neuroadaptations resulting in excessive incentive salience attribution to drug-associated cues, or 'incentive-sensitization.' Here, the excessive incentive salience of the cue provokes overwhelming cue-triggered drug wanting, evidenced by conscious drug craving as well as unconscious compulsive behavior. These findings are consistent with

the behavior of human addicts who have developed tolerance to hedonic drug effects nonetheless report strong desires to take drugs. Behavioral evidence of incentive sensitization in rodents is observed as increases in sign-tracking, Pavlovian-instrumental transfer, and conditioned reinforcement by drug-associated cues after repeated drug treatment. Sensitization of the locomotor response to psychostimulants, which is accompanied by heightened DA release, is also frequently used as an index of incentive-sensitization. Behavioral sensitization is long-lasting and modulated by context, suggesting persistent changes in the mesolimbic system that interact with associative contextual memory (Berke and Hyman, 2000; Robinson and Berridge, 2008).

The prediction error theory has somewhat supplanted incentive salience in popularity, but the authors who introduced the concept have maintained their stance that incentive salience attribution best describes the function of DA, and even suggest that apparent prediction-error behavior is actually an artifact of animals being consistently tested in the same motivational state (i.e. food or water-deprived) (Berridge, 2012). Incentive salience is distinguished from common-currency cue values described in TD models, in that it is a dynamic computation that includes the state of the animal, such that an animal would attribute greater incentive salience to a food-predictive cue when hungry than when full, but a TD model considers the cue value fixed (Berridge, 2012). For example, high-salt solutions are aversive unless the animal is in a salt-depleted state, in which case it becomes hedonic. Animals that have learned that a lever

predicts oral delivery of aversively salty solution will lever-press when salt depleted, despite the fact that they have only ever experienced salt as aversive (Robinson and Berridge, 2013). RPE theory does not predict this behavior, as the last cached reward value of the cue before salt depletion was low, and a rewarding experience with salt would be required to evoke a “better than predicted” error to salt delivery and drive the necessary learning to update the cue value. It is theorized that these incentive salience signals are computed when cue-reward associations are scaled by inputs representing the animal’s internal state (Berridge, 2012; Zhang et al., 2009). If DA neurons do in fact signal incentive salience, they must have access to model-based information about the nature of reward associated with the cue, and model-based input from the orbitofrontal cortex has been reported (Takahashi et al., 2011).

Habits, Compulsions and Executive Function

Although the proposed underlying processes are somewhat different, persistent RPE and excessive incentive salience attribution both result in drugs becoming highly valued end goals. As a result, individuals choose drug-seeking over other pressing concerns when deciding among behavioral options (Montague et al., 2004). Individuals also continue to take drugs in spite of negative consequences, which may be influenced by drug-induced impairments in top-down inhibition (Cohen et al., 2002). These interpretations both assume that drug use results after a PFC-dependent economic decision-making process,

which evaluates drugs as the highest-value option among goals and judges the drug value to outweigh the costs paid in effort and/or punishment. Here, 'compulsive' drug use still requires a decision, although the decision is likely unconscious. However, studies indicate that prolonged drug use shifts the underlying control of drug-taking from a goal-directed process to a habitual response (Everitt and Robbins, 2016). Learning of habitual responses, which are cue-evoked, are dependent on phasic DA bursting (Wang et al., 2011). In an operant reinforcement cue-action-outcome scenario, actions are considered goal-directed if they are sensitive to changes in the outcome. Habitual responses to cues will continue in spite of the outcome being devalued, punished, or omitted entirely. This shift to habitual responding is not unique to addiction; for example, people will habitually flick a light switch when walking into a room, despite having witnessed the bulb burn out (Yin and Knowlton, 2006). In experiments investigating both natural and drug reinforcement, it was found that dorsomedial striatum-PFC activity is important to maintain early operant training, but training that is prolonged and insensitive to changes in outcome recruits the dorsolateral striatum (Everitt and Robbins, 2016). A criticism of the habitual view of compulsive drug-taking is that human addicts display complex drug-seeking behavior that is too flexible and cognitively demanding to be explained by stimulus-response habits (Robinson and Berridge, 2008). This disagreement is primarily due to different interpretations of the term 'compulsive drug use'. Habitual drug taking likely comes into play when an individual is attempting to

limit intake of a readily available drug (e.g., mindlessly lighting a cigarette or grabbing a beer from the refrigerator), while an individual that has no ready access to drugs will likely experience craving and intensely motivated drug-seeking. Either scenario is a form of 'lost control' over drug-taking. Roughly 20% of rats and humans will actively continue drug-taking in the face of direct punishment, suggesting that habitual responding may be especially problematic in a subpopulation of individuals (Everitt and Robbins, 2016).

IMPORTANCE OF PAVLOVIAN CUE-DRUG ASSOCIATIONS

Addiction has been described as a 'disease of learning and memory,' and there are likely several associative memory processes mediated by the PFC, NAc, and amygdala that are involved in addiction (Hyman et al., 2006). A shared element of the theories described above is DA-dependent learning of drug-associated cues. Whether they evoke a state of intense motivation to seek and consume drugs, bias decision-making towards continued drug use at the expense of daily responsibilities, invigorate ongoing drug-seeking actions, or facilitate habit formation, pathologically strong Pavlovian cue-drug associations made in the early stages of drug use are indispensible for the transition to addiction, and cue-triggered craving and drug-seeking leads to relapse. Conditioned phasic DA release to reward-associated cues, which develops with Pavlovian conditioning, is a likely neurobiological correlate of associative learning

of the cue's rewarding valence. Consistent with this idea, blockade of phasic DA release impairs expression of previously acquired Pavlovian conditioned place preference (Whitaker et al., 2013) and cue-driven invigoration of reward seeking actions (Wassum et al., 2012).

PLASTICITY IN VTA DA NEURONS

The importance of phasic DA signals for reward learning and addiction has sparked investigation of plasticity mechanisms in VTA DA neurons in an attempt to find cellular correlates of drug-induced neuroadaptations and associative learning. LTP and LTD of AMPAR transmission, LTP of GABAergic transmission, and LTP of NMDAR transmission have all been described. Interestingly, each of these plasticities interacts with stress.

AMPAR Synaptic Plasticity

LTP of AMPARs in VTA DA neurons was first described in vitro, evoked with high-frequency stimulation and, later, via a spike-timing induction protocol (Bonci and Malenka, 1999; Liu et al., 2005). AMPAR-LTP induction is NMDAR-dependent and its expression requires upregulation of GluR1-containing AMPARs (Dong et al., 2004). Further experiments revealed experience-dependent AMPAR plasticity, as a single treatment with cocaine, amphetamine, morphine, nicotine, or ethanol or even a single forced swim stress session is

sufficient to induce LTP that persists for roughly one week. Experience-induced LTP occludes further induction in slices taken from treated animals (Saal et al., 2003; Ungless et al., 2001). Stress and drugs appear to induce AMPAR LTP via separate pathways. GR signaling is necessary for stress-induced but not drug-induced plasticity, and pharmacological GR activation alone is sufficient for induction (Daftary et al., 2009). The role of AMPAR-LTP in reward learning and/or addiction-related behavior is unclear. In GluR1-KO mice that cannot express AMPAR LTP, behavioral sensitization was unaffected. Cocaine CPP was impaired in the KOs; however, the use of a global KO here cannot definitively confirm a role for GluR1 in VTA (Dong et al., 2004). Stuber et al found that as animals acquired Pavlovian conditioned approach for sucrose reward, AMPAR: NMDA transiently increased, but reversed before acquisition of the CR was complete (Stuber et al., 2008). Reversal may be mediated by mGluR-dependent AMPAR LTD, which is mGluR-dependent and involves exchange of higher-conductance GluR2-lacking AMPARs for lower-conductance GluR2-containing receptors (Bellone and Luscher, 2006; Mameli et al., 2007). The time course of AMPAR potentiation is incompatible with long-term storage of Pavlovian associations, and the measurable increase in macroscopic AMPAR-mediated current suggests a lack of input-specificity. AMPAR potentiation seems to correlate with periods of high phasic DA activity, but its functional significance remains elusive.

Inhibitory Synaptic Plasticity

GABAergic input to VTA DA neurons is also plastic. High-frequency excitation of DA neurons generates nitric oxide, a retrograde messenger that potentiates GABA release from presynaptic terminals (Nugent et al., 2007). Interestingly, the modulation of GABA LTP by experience is opposite of what is found with AMPA LTP. Both stress and drugs of abuse (with the exception of ethanol, which potentiates GABA transmission (Melis et al., 2002)) block induction of LTP-GABA. GABA LTP impairment is present 24 hours after drug treatment but reverses in under 5 days and therefore is more short-lived than AMPAR plasticity (Niehaus et al., 2010). The combined effects of AMPAR potentiation and impaired GABA LTP are expected to increase DA neuron excitability after drug or stress experience (Polter and Kauer, 2014). *In vivo* recordings of DA neuron activity have found that tonic firing is elevated for 1-3 days after psychostimulant experience, whereas bursting is increased only on the first day of withdrawal (Marinelli et al., 2003). These results support the idea of increased neuronal excitability, but the effect is more transient than would be expected given that AMPA potentiation persists for 1 week.

NMDAR Synaptic Plasticity

LTP of NMDAR transmission in VTA DA neurons is an attractive candidate for long-term memory of reward-associated cues. NMDAR activation drives the transition from tonic firing to bursting in DA neurons (Chergui et al., 1993; Grace

and Bunney, 1984; Overton and Clark, 1997); therefore, LTP of cue-driven NMDAR input to DA neurons may underlie the conditioned bursting observed after Pavlovian conditioning. Induction of NMDAR-can be achieved by protocol that mimics putative activity during a Pavlovian conditioning paradigm by repeatedly pairing cue-like synaptic stimulation, which activates mGluRs, with reward-like bursting of the DA neuron (Harnett et al., 2009). This plasticity is input-specific and sensitive to the relative timing of synaptic stimulation and burst. NMDAR-LTP induction requires facilitation of burst-evoked Ca^{2+} entry by IP_3R-mediated Ca^{2+}-induced Ca^{2+}release (CICR) from intracellular stores, and the magnitude of facilitation correlates with the magnitude of LTP. Here, the inositol-1,4,5-triphosphate receptor (IP_3R) detects the coincidence of IP_3 generated by synaptic stimulation and burst-driven Ca^{2+} entry and gates LTP induction via Ca^{2+} efflux from the endoplasmic reticulum. The downstream mechanisms of NMDAR-LTP expression have not been elucidated. A necessary role has been identified for L-type Ca^{2+} channels, specifically the low-threshold subtype $Ca_v1.3$ (Degoulet et al., 2015). LTCCs are necessary for NMDAR LTP induction but do not contribute to burst-evoked Ca^{2+} signals or their facilitation by IP_3R-mediated CICR. The precise mechanism of NMDAR LTP expression is unresolved, but the locus of expression is postsynaptic and pharmacological data do not indicate a change in receptor subtypes (Harnett et al., 2009).

Unlike AMPARs, global NMDAR expression is not sensitive to psychostimulant treatment (Ungless et al., 2001). However, unitary NMDA

EPSCs evoked by two-photon glutamate uncaging were reduced after cocaine treatment, suggesting a functional NMDAR LTD at synaptic sites (Mameli et al., 2011). These combined findings suggest redistribution of NMDARs, although it has not been tested whether the NMDARs migrate to extrasynaptic sites.

Prolonged exposure to ethanol, psychostimulants, or an impoverished environment increases IP_3R sensitivity to IP_3 via a PKA-dependent mechanism, effectively lowering the threshold for coincidence detection and therefore, NMDAR LTP induction (Ahn et al., 2010; Bernier et al., 2011; Whitaker et al., 2013). These findings indicate that drug experience and environmental factors can regulate the sensitivity of the synaptic plasticity induction machinery in a metaplastic manner.

CALCIUM SIGNALING IN DA NEURONS

In DA neurons, Ca^{2+} signals shape cell firing activity and participate in the induction of synaptic plasticity. These signals can be initiated DA by influx through voltage-gated Ca^{2+} channels (VGCCs) or Ca^{2+}-permeable glutamate receptors and amplified by CICR from stores within the endoplasmic reticulum. Tonic firing frequency is regulated by VGCC Ca^{2+} influx during the action potential, which recruits small-conductance Ca^{2+} -activated potassium (SK) channels to produce a rate-limiting afterhyperpolarization (Wolfart et al., 2001). SK channels are voltage-independent; therefore voltage-clamp recordings of SK

currents evoked by incompletely clamped APs are effective indicators of AP-mediated Ca^{2+} entry (Cui et al., 2007).

CICR is mediated by IP_3Rs and ryanodine receptors (RyRs), which are coexpressed on the same store in DA neurons (Morikawa et al., 2000). Synaptic activation of G_q-coupled receptors, such as mGluRs, muscarinic acetylcholine receptors, and α1 adrenergic receptors stimulates phospholipase C, which then generates IP_3 to trigger CICR via IP_3Rs. IP_3Rs contain separate stimulatory and inhibitory Ca^{2+} binding sites, and IP_3 is thought to promote receptor activity by controlling relative access to the stimulatory *vs.* inhibitory sites (Taylor and Laude, 2002). Strong mGluR stimulation can additionally activate RyRs via a non-canonical coupling to mGluR. The resulting wave of store Ca^{2+} release can pause tonic firing and attenuate burst frequency via SK-mediated hyperpolarization (Morikawa et al., 2003).

AP-triggered Ca^{2+} signals can be amplified by CICR (Berridge, 1998), which may be induced either by the AP Ca^{2+} influx itself or by interaction with IP_3 generated prior to the AP by G_q-coupled receptor activity. In DA neurons, tonic firing activity partially inactivates IP_3Rs, leading to selective amplification of burst-evoked Ca^{2+} signals. In tonically firing DA neurons, Ca^{2+} influx from single APs cannot trigger CICR its own, nor can it be facilitated by IP_3. In contrast, under the same conditions, the larger Ca^{2+} influx from a burst of APs can trigger CICR that is further facilitated by IP_3 (Cui et al., 2007). This selective facilitation of burst-evoked Ca^{2+} signals is essential to the IP_3R's role as a coincidence detector for

prior presynaptic stimulation and bursting in NMDAR LTP induction (Harnett et al., 2009).

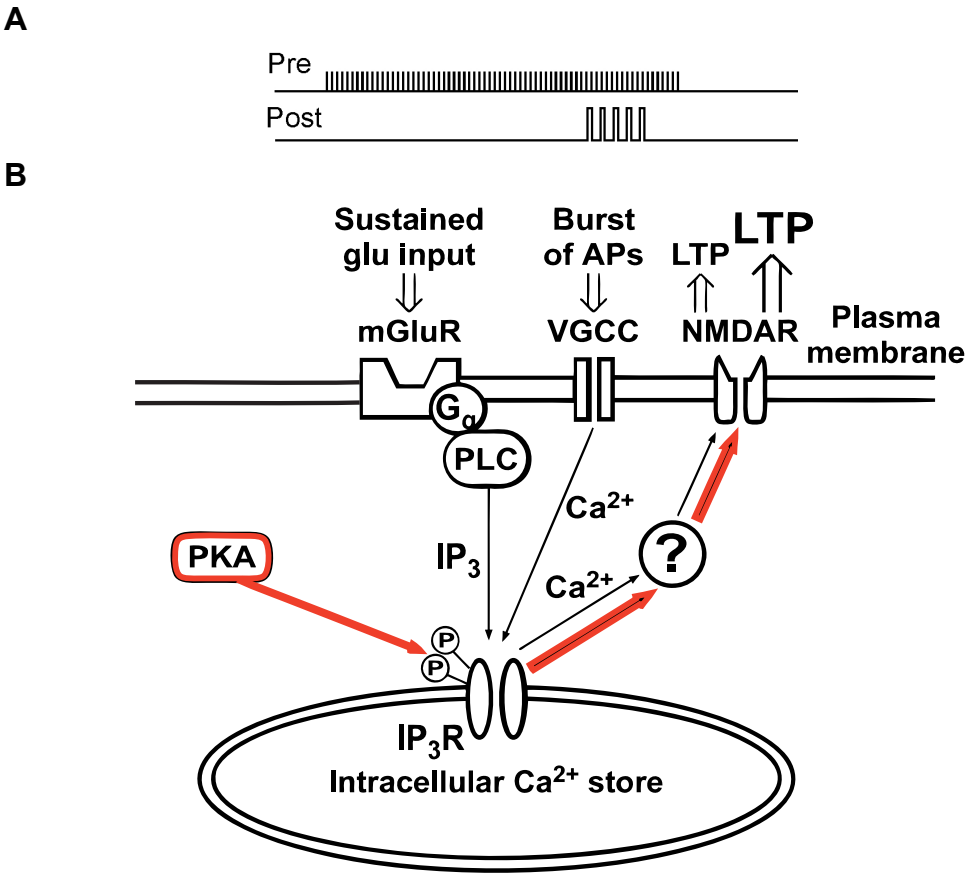

Figure 4. NMDAR LTP and metaplasticity pathways in VTA DA neurons

LTP of NMDAR transmission is induced by repeatedly pairing cue-like sustained presynaptic stimulation with a reward-like burst of action potentials (APs). (**A**) Schematic of LTP induction protocol. (**B**) Molecular pathways underlying LTP and metaplasticity (in red). Sustained synaptic stimulation activates mGluRs to generate IP_3. Burst-evoked Ca^{2+} signals are facilitated by IP_3R-mediated CICR. The resulting Ca^{2+} efflux potentiates NMDAR transmission by an unknown mechanism. PKA phosphorylation enhances IP_3R affinity for IP_3, allowing greater CICR to be achieved by the same amount of synaptic stimulation.

RATIONALE, HYPOTHESIS, AND AIMS

It has been well-documented that stress exposure increases acquisition of addiction-related behaviors tested across every class of abused drug (Becker et al., 2011; Boyson et al., 2014; Burke et al., 2011; Der-Avakian et al., 2007; Goeders and Guerin, 1994; Hadaway et al., 1979; Han et al., 2015; Kreibich et al., 2009; Piazza et al., 1990; Shaham and Stewart, 1994). The general interpretation of these findings has been that stress increases the rewarding effect of drugs. This idea is supported by findings that prior stress cross-sensitizes DA release to psychostimulants and opioids; however there is insufficient data on stress cross-sensitization with other classes of drugs to extend this interpretation to them. An alternative explanation of stress's effect on addiction-related behaviors is that stress enhances the mechanisms underlying Pavlovian learning of drug-associated cues independently of changes in drug reward. Stress has been shown to enhance Pavlovian fear conditioning in this manner by inducing metaplasticity in basolateral amygdala neurons (Suvrathan et al., 2013). It should be noted that enhanced cue learning and enhanced drug reward are not mutually exclusive and likely act in tandem.

Studies form our lab have described plasticity of NMDAR transmission that may underlie learning the rewarding value of drug-associated cues (Harnett et al., 2009). It has been shown previously that drug experience and social isolation induce metaplasticity, sensitizing the LTP induction pathway (Ahn et al.,

2010; Bernier et al., 2011; Whitaker et al., 2013). In light of these findings and the known metaplastic effects of stress in other brain regions (Schmidt et al., 2013), this project sought to test the hypothesis that stress induces metaplasticity of NMDAR transmission in DA neurons. The social defeat model of stress was chosen because (1) it has face validity for human stress, which is frequently social in nature, (2) it has predictive validity for the high comorbidity of depression in individuals with substance use disorders (Krishnan et al., 2011; Miczek et al., 2008). The first aim of this project was to investigate the effect of repeated social defeat stress on Ca^{2+} signaling and NMDAR LTP in DA neurons. The second aim of this project was to investigate the impact of repeated social defeat stress on the acquisition of psychostimulant CPP.

Chapter 2: Materials and Methods

ANIMALS

All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and were approved by the University of Texas at Austin Institutional Animal Care and Use Committee. Sprague-Dawley rats (Harlan Laboratories) were housed in groups of 2-3 on a 12 hour light/dark cycle (lights on at 7:00 AM). Food and water were provided *ad libitum*.

RESIDENT-INTRUDER SOCIAL DEFEAT

Twelve-week-old male 'resident' rats were vasectomized and pair-housed with 6-week-old females to encourage territorial behavior. Residents were screened for aggression by introducing a young male intruder to the home cage. Residents that displayed aggressive behavior such as grooming, pinning, or biting within 1 min of intrusion were selected for social defeat experiments. Residents were used for multiple experiments as long as they continued to display aggression. Female cage mates were rotated among residents. 'Intruders' and controls were 4-5 week-old males housed in groups of 2-3. For defeat sessions, residents and intruders were taken to a darkened procedure room at the end of the dark cycle. Females were transferred to a holding cage.

Intruders were introduced to residents' home cages, and the rats were allowed 5 minutes of direct contact. After the direct interaction, a perforated Plexiglass barrier was inserted for 25 minutes to physically separate animals while allowing sensory contact. All animals were inspected for injury after the session. For repeated defeat, intruders underwent one session daily with a novel resident. Handled controls were taken to a darkened procedure room and placed in novel, clean cages for 30 minutes. Naive controls were undisturbed in the colony until sacrifice. After initial experiments had confirmed no effect of transport and handling, data from handled and naive controls were pooled into a 'Control' group.

IN VIVO DRUG TREATMENT

All drug and vehicle solutions were administered via i.p. injections 1 mL/kg in volume. Mifepristone (40 mg/mL, Tocris) was dissolved in 30% propylene glycol plus 1% Tween-20 in sterile 0.9% saline (Hospira). Corticosterone (2.5, 5, and 15 mg/mL) was dissolved in 30% propylene glycol plus 1% Tween-20 in sterile 0.9% saline. Cocaine-HCl (5 mg/mL, Sigma-Aldrich) was dissolved in sterile 0.9% saline.

ELECTROPHYSIOLOGY

Rats (5-6 weeks old unless otherwise specified) were anesthetized with isoflurane and decapitated. Horizontal midbrain slices containing the VTA were

cut with a vibrating microtome (Thermo Scientific) in ice-cold oxygenated saline containing (mM): 205 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 7.5 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose, and 25 $NaHCO_3$. Slices recovered for 1 hour at 34° C in oxygenated artificial cerebrospinal fluid containing (mM) 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose, and 21.4 $NaHCO_3$. For recordings, slices were perfused with the same artificial cerebrospinal fluid at 34°C. Whole-cell recordings were made from neurons in the lateral VTA (50–150 μm from the medial border of the medial terminal nucleus of the accessory optic tract). Pipettes were filled with (mM): 115 K- methylsulfate, 20 KCl, 1.5 $MgCl_2$, 10 HEPES, 0.025 EGTA, 2 Mg-ATP, 0.2 Na_2- GTP and 10 Na_2-phosphocreatine, pH 7.25. Putative dopamine neurons were identified by spontaneous firing of broad APs (>1.2 ms) at 1-5 Hz in cell-attached configuration and large I_h currents (>200 pA response to a 1.5 s hyperpolarizing step from -62 mV to -112 mV) in whole-cell configuration. These criteria (lateral VTA location with slow spontaneous firing rates and large I_h) select for lateral accumbens shell-projecting DA neurons (Lammel et al., 2011). Cells were voltage-clamped at –62 mV (corrected for –7 mV liquid junction potential). Recordings were discarded if series resistance increased above 20 MΩ or input resistance dropped below 200 MΩ. Signals were acquired with a Multiclamp 700B amplifier (Molecular Devices), digitized at 10-20 KHz, and filtered at 2-10 KHz. Data acquisition and analysis were performed with Axograph X (Axograph Scientific).

Firing Rate

Loose-patch recordings (~20 MΩ seal) were made using pipettes filled with 150mM NaCl to monitor dopamine neuron firing. Spontaneous action potentials were recorded with the amplifier in I=0 mode (Perkins, 2006). Action potentials were detected and counted with the event detection utility in Axograph X.

Burst-Evoked Calcium Transients

Bursts of 5 incompletely clamped APs at 20 Hz were evoked by 2ms depolarizing steps from - mV to 0 mV. AP-induced Ca^{2+} entry through voltage-gated Ca^{2+} channels or release from intracellular stores activates small-conductance Ca^{2+}-sensitive potassium (SK) channels to produce an outward tail current, termed $I_{K(Ca)}$, that is completely blocked by tetrodotoxin or apamin and therefore a metric of AP-induced Ca^{2+} transients. Burst-evoked $I_{K(Ca)}$ charge transfer was calculated as the sum of the time integral of the tail current following each AP within the burst.

Ultraviolet Flash Photolysis

Cells were loaded with caged IP_3 (50-400 μM) through the recording pipette. Recordings began 15 min after break-in to allow equilibration of IP_3 in the cytosol. A brief UV flash (~1 ms) was applied with a xenon arc lamp driven by a photolysis system (Cairn Research) to rapidly uncage IP_3. The UV flash was

focused through a 60X objective onto a 350 μm area surrounding the recorded neuron. Photolysis of caged compounds is proportional to the UV flash intensity (McCray et al., 1980). Therefore, the concentration of photolyzed IP_3 (expressed in μJ•μM) was defined as the product of the caged IP_3 concentration in the pipette (μM) and the UV flash intensity (μJ) measured at the focal plane of the objective with an optical energy meter (ThorLabs). Flash-evoked $I_{K(Ca)}$ was measured as a readout of IP_3R-mediated Ca^{2+} release from intracellular stores.

NMDAR LTP

Synaptic stimuli were delivered with a bipolar tungsten stimulating electrode (100 μm tip separation) placed 50-100 μm rostral to the VTA. To isolate and enhance NMDAR EPSCs, recordings were performed in ACSF containing .1 mM Mg^{2+}, 20 μM glycine, 10 μM DNQX, 100 μM picrotoxin, 50 nM CGP54626, and 100 nM sulpiride. NMDAR EPSCs were monitored every 20 seconds. The LTP induction protocol consisted of photolytic application of 50 μJ•μM IP_3 50 ms prior to the simultaneous delivery of synaptic stimulation (20 stimuli at 50 Hz) and a 20 Hz burst of 5 APs, repeated 10 times every 20 s. LTP magnitude is reported as the average EPSC amplitude (15 sweeps) 40 min post-induction divided by the average EPSC 5 min pre-induction.

CONDITIONED PLACE PREFERENCE

A CPP box (Med Associates) consisting of two distinct compartments joined by a small middle chamber was used for conditioned place preference. One compartment had a mesh floor with white walls, while the other had a grid floor with black walls. A discrete cue (ceramic weight painted in a contrasting color to the walls) was placed in the rear corner of each compartment to further differentiate the contexts. 5-week old rats underwent 5 days of social defeat or control procedure. One day after the last stress or control session, rats were pretested for initial side preference by allowing unrestricted exploration of the entire CPP box for 15 min. The percentage of time spent in each compartment was determined after excluding the time spent in the middle chamber. Rats with an initial side preference >60% were excluded. The following day (48 hours after the last stress or control procedure), rats were given a saline injection (1 ml/kg, i.p.) in the morning and confined to one compartment for 10 min. In the afternoon of the same day, rats were given a cocaine injection (5 mg/kg, i.p.) and confined to the other compartment for 10 min. Compartment assignment was counterbalanced such that animals had, on average, ~ 50% initial preference for the drug-paired side in the pretest, i.e., approximately half of animals received drug in the preferred compartment, and half in the non-preferred compartment. A 15 min posttest was performed 1 day after conditioning to assess conditioned place/cue preference. The CPP score was calculated as follows: ΔPreference = (% time spent in drug-paired side during post-test) – (% time spent in drug-paired

side during pre-test). The experimenter performing behavioral testing was blind to animal treatment.

DATA ANALYSIS

Statistical tests were performed with Prism (GraphPad). Data are expressed as mean ±SEM. Statistical significance was assessed with Student's t-test, 1-way ANOVA, or 2-way ANOVA, and post hoc comparisons were performed when applicable. P-values <0.05 were considered significant.

Chapter 3: Results

REPEATED SOCIAL STRESS INCREASES MGLUR-MEDIATED FACILITATION OF BURST-EVOKED CA^{2+} SIGNALS.

NMDAR-LTP induction requires facilitation of burst-evoked Ca^{2+} entry by IP_3R-mediated release of Ca^{2+} from intracellular stores, and the magnitude of facilitation correlates with the magnitude of LTP induced (Harnett et al., 2009). To assess the effect of stress on facilitation of burst Ca^{2+} signals, whole-cell voltage-clamp recordings were performed in midbrain slices prepared from rats that were naïve, handled, or socially defeated for 1, 5, or 10 consecutive days (see Methods chapter for details). Animals were killed 24-48 hours after the final behavioral treatment. Burst-evoked Ca^{2+} signals were assessed by measuring the burst-evoked Ca^{2+}-activated SK current (burst $I_{K(Ca)}$) (see Methods chapter for details). Group I metabotropic glutamate receptors were pharmacologically activated by bath perfusion of agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG) (Figure 5A). The facilitating effect of DHPG on burst-evoked Ca^{2+} signals was calculated and compared among groups (Figure 5B). The magnitude of DHPG effect on $I_{K(Ca)}$ was significantly larger in animals that underwent 5 or 10 days of social defeat stress compared to naïve controls and handled controls ($p<.05$, Bonferroni post-hoc test). A single social defeat session did not significantly increase the DHPG effect relative to either control group. There was no significant difference between naïve controls and handled controls, therefore

data from naïve and handled animals were pooled into a single 'Control' group in subsequent experiments. The effect of stress on $I_{K(Ca)}$ facilitation plateaus by 5 days, as there was no significant difference between groups receiving 5 or 10 days of social defeat. Basal burst $I_{K(Ca)}$ was consistent across groups, indicating that stress treatment does not alter AP-associated Ca^{2+} influx by voltage-gated Ca^{2+} channel-mediated (Figure 5C).

To examine the persistence of stress-induced changes in $I_{K(Ca)}$ facilitation, the interval between the last social defeat session and animal sacrifice was prolonged to 10 and 30 days (Figure 5D). Steady, spontaneous reversal of the stress effect on $I_{K(Ca)}$ facilitation was observed. DHPG-facilitation of $I_{K(Ca)}$ remained significantly elevated 10 days post-stress ($p<.05$, Bonferroni post hoc test) but by 30 days post-stress did not differ from age-matched controls. These data indicate a window of time following stressful experience during which the NMDAR-LTP induction mechanism is sensitized.

Group I mGluR activation also evokes a small, sustained inward current in DA neurons that is independent of Ca^{2+} mobilization (Guatteo et al., 1999). This DHPG-induced inward current was also consistent across groups, indicating that stress-mediated changes in $I_{K(Ca)}$ facilitation occur downstream of mGluR (Figure 5E).

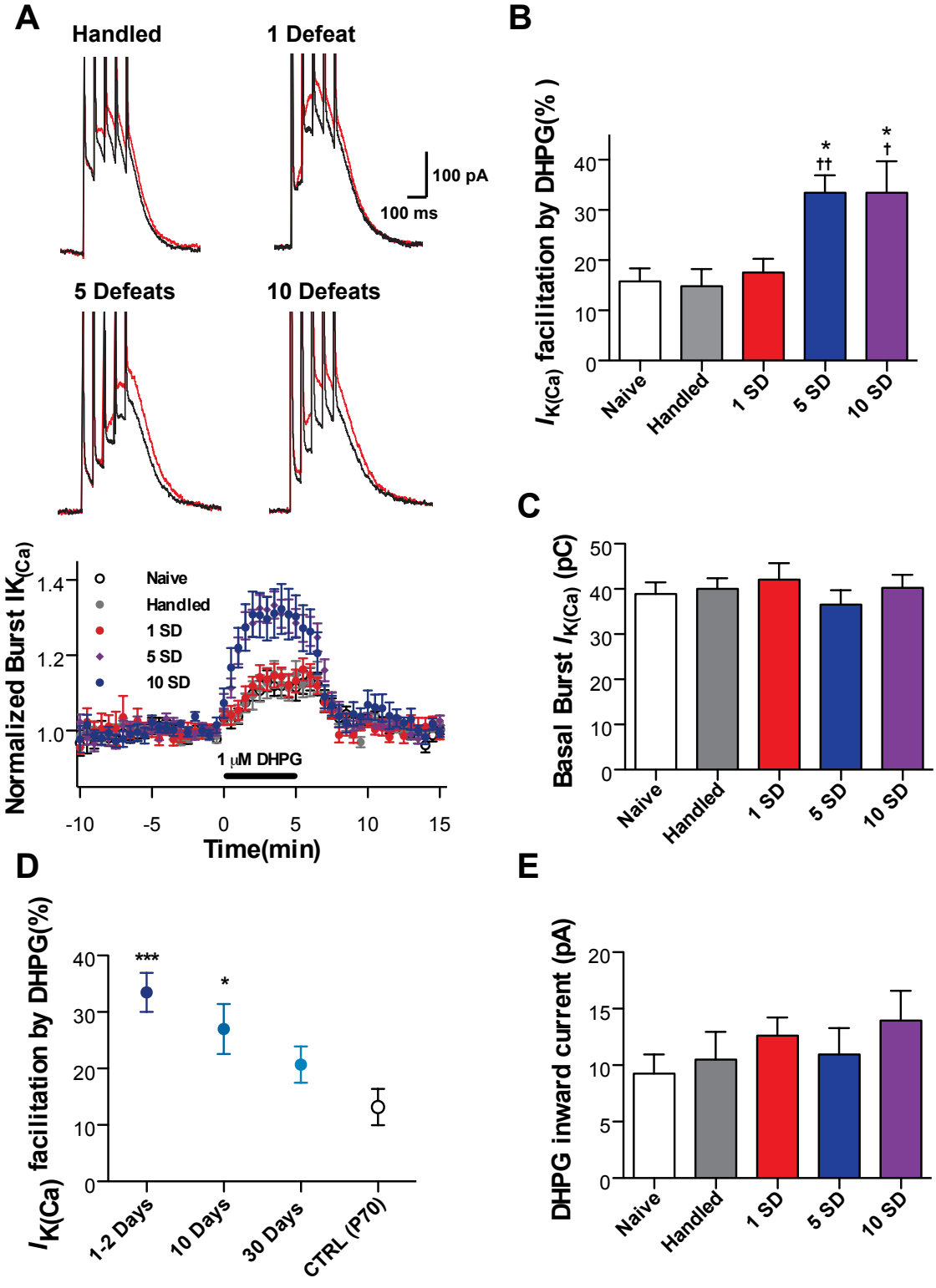
A
Handled
1 Defeat
100 pA
100 ms
5 Defeats
10 Defeats
Normalized Burst IK(Ca)
Naive
Handled
1 SD
5 SD
10 SD
1 μM DHPG
Time(min)
B
IK(Ca) facilitation by DHPG(%)
Naive
Handled
1 SD
5 SD
10 SD
C
Basal Burst IK(Ca) (pC)
D
IK(Ca) facilitation by DHPG(%)
1-2 Days
10 Days
30 Days
CTRL (P70)
E
DHPG inward current (pA)

Figure 5. mGluR-dependent facilitation of burst-evoked Ca^{2+} signals is enhanced after repeated social defeat.

(**A**) Example traces (top) and summary time graph (bottom) of burst $I_{K(Ca)}$ illustrating the facilitating effect of 5min bath-perfused DHPG (1μM) in VTA DA neurons from rats there were naïve, handled for 5 days, or underwent social defeat for 1, 5, or 10 days. Inward action currents have been removed for clarity. (**B**) Summary of percent burst $I_{K(Ca)}$ facilitation by DHPG (naïve=20 from 12 rats; handled = 20 from 13 rats; 1 defeat = 19 from 11 rats; 5 defeats = 21 from 13 rats; 10 defeats = 19 from 10 rats, $F_{4,94}$=6.19, p<.001, 1-way ANOVA. **p*<.05 compared to naïve; † *p*<.05 compared to handled; ††*p*<.01 compared to handled, Bonferroni post hoc test). (**C**) Summary of basal burst $I_{K(Ca)}$ ($F_{4,94}$=.49, *p*=0.74, 1-way ANOVA). (**D**) Summary graph depicting DHPG-induced burst $I_{K(Ca)}$ facilitation after varying intervals of time after 5 social defeat sessions. The 1-2 day group data were taken from the initial experiment depicted in panels A and B. Controls were age-matched to the 30 day post-stress group. (1-2 days= 21 from 13 rats; 10 days=18 from 8 rats; 30 days= 16 from 9 rats; age-matched control = 17 from 7 rats. $F_{3,66}$=5.87, *p*<.01, 1-way ANOVA). **p*<.05, ****p*<.001 compared to control; Bonferroni multiple comparison post hoc test. (**E**) Summary of DHPG-induced inward current ($F_{4,94}$=.70, *p*=0.56, 1-way ANOVA).

REPEATED SOCIAL STRESS INDUCES PKA-DEPENDENT SENSITIZATION OF IP_3RS.

To ascertain if the stress-mediated increase in DHPG effect was due to increased IP_3R sensitivity, IP_3 concentration-response curves were generated (Figure 6A). Caged IP_3 was loaded into DA neurons through the recording pipette and varying concentrations of IP_3 were uncaged with brief flashes of UV light (see Methods for details). IP_3R-mediated Ca^{2+} release was assessed by measuring peak flash-evoked SK current, I_{IP3}. The average IP_3 concentration-response curve was significantly left-shifted ($p<.001$, Bonferroni post hoc test) in animals that underwent 5 social defeats compared to controls (Figure 6B). Accordingly, the EC_{50} of IP_3 was significantly smaller in defeated rats ($p<.05$, Bonferroni post hoc test) (Figure 6C). Maximal I_{IP3} did not differ between groups, consistent with a change in receptor affinity for IP_3 but not IP_3 efficacy (Figure 6D).

PKA phosphorylation increases IP_3R sensitivity in expression systems (Wagner et al., 2008), and this effect has been demonstrated in VTA DA neurons (Bernier et al., 2011; Harnett et al., 2009). To determine whether PKA phosphorylation underlies the stress-mediated IP_3R sensitization, the effect of a selective peptide inhibitor of PKA, PKI-(6-22)-amide (PKI), was tested on the IP_3 concentration-response in cells from stressed and control animals (Figure 6B). PKI and caged IP_3 were co-loaded through the recording pipette and dose-responses were obtained as described above. There was no significant difference in EC_{50} of IP_3 between groups in PKI-loaded neurons, indicating that

the left-shifted dose-response observed in stressed animals is maintained by PKA-dependent phosphorylation of the IP_3R (Figure 6C). Moreover, PKI treatment did not right-shift the IP_3 dose-response in the control group, suggesting low PKA-dependent phosphorylation under basal conditions. Maximal currents did not differ across groups, indicating that stress nor PKI affected IP_3 efficacy. Together, these data demonstrate that repeated social stress sensitizes IP_3Rs via a PKA-dependent mechanism.

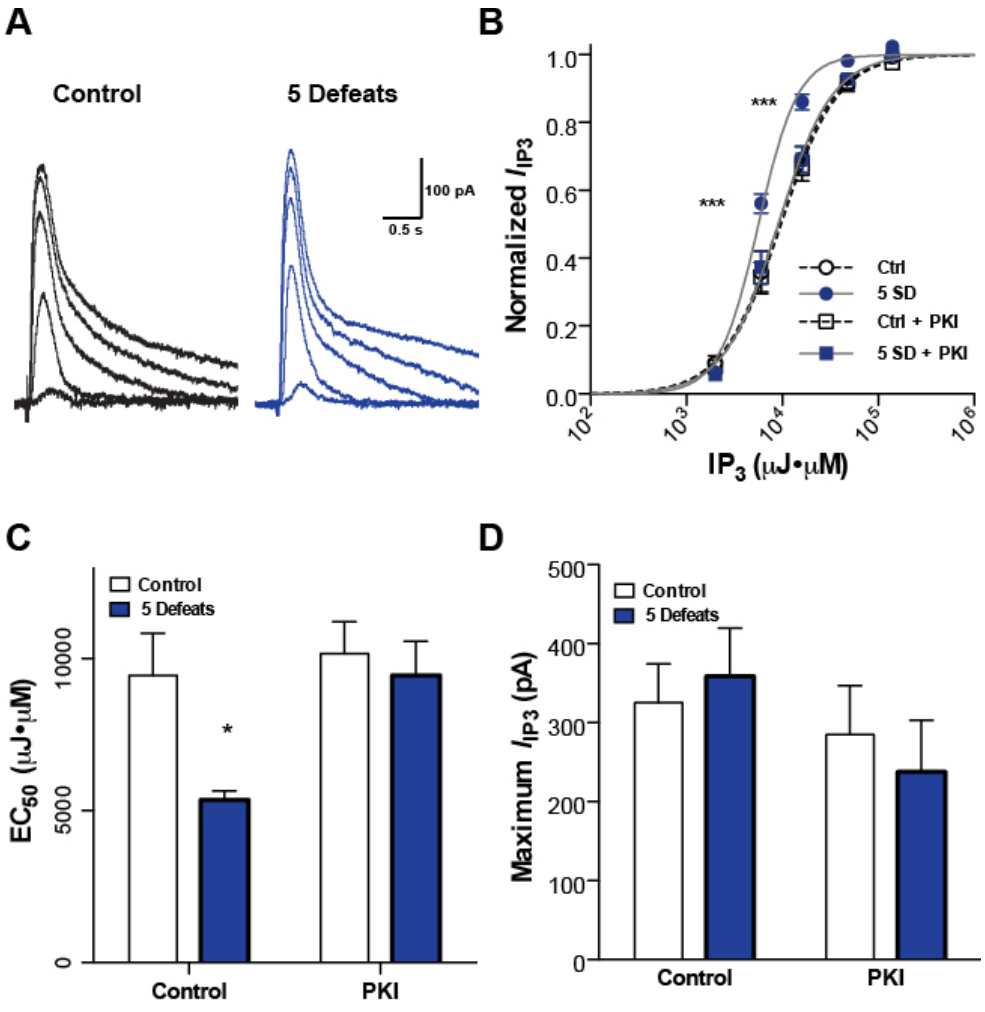

Figure 6. PKA activity maintains increased IP_3R sensitivity after social defeat

(**A**) Example traces of I_{IP3} evoked by photolytically uncaging varying concentrations of IP_3 in VTA DA neurons from a control and a defeated rat. IP_3 concentrations (in μJ•μM) are 2000, 6000, 16000, 48000, 140000. (**B**) Averaged IP_3 dose-response curves from control and defeated rats, with and without intracellularly loaded PKA inhibitor PKI-(6-22)-amide. Data are normalized by maximal within-cell current response to IP_3. Lines represent logistical equation fits. (control= 12 from 8 rats; 5 defeats = 12 from 7 rats; Control + PKI= 14 from 9 rats; 5 defeats+ PKI= 14 from 8 rats. defeat: $F_{3,196}$=4.88, p<.01; IP_3 concentration: $F_{4,196}$=1214, p<.001; defeat x IP_3 concentration: $F_{12,196}$=4.42, p<.001; mixed 2-way ANOVA.) ***p<.001, Bonferroni post-hoc test. (**C**) Summary of EC_{50} IP_3 concentrations (defeat: $F_{1,49}$=5.11, p<.05; PKI: $F_{1,49}$=5.11, p<.05; defeat x PKI: $F_{1,49}$=2.54, p=.12; 2-way ANOVA). *p<.05, Bonferroni post hoc test. (**D**) Summary of maximal IP_3-evoked current (defeat: $F_{1,49}$=.012, p=0.91; PKI: $F_{1,49}$=1.76, p=.19; defeat x PKI: $F_{1,49}$=.435, p=.51; 2-way ANOVA).

REPEATED SOCIAL STRESS ENHANCES LONG-TERM POTENTIATION OF NMDAR EPSCS

In DA neurons, IP_3Rs gate NMDAR-LTP induction by detecting the coincidence of IP_3 generation and burst-evoked Ca^{2+} entry (Harnett et al., 2009). Therefore, the effect of repeated social defeat, which sensitizes IP_3Rs, was tested on induction of NMDAR-LTP. The LTP induction protocol consisted of uncaging a low concentration of IP_3 (250 μJ•μM) 50ms prior to pairing a burst (5 incompletely clamped APs at 20 Hz) with a brief train of synaptic stimuli (20 pulses at 50 Hz) (Figure 7A). This procedure was repeated every 20s for a total of 10 pairings. The concentration of IP_3 used in the induction protocol produces little to no detectable I_{IP3}, but facilitates burst-evoked $I_{K(Ca)}$, and facilitation was significantly larger in cells from defeated animals($p<.01$, unpaired t-test) (Figure 7B). The LTP induction protocol produced a very small magnitude of LTP in cells from control animals, but significantly larger LTP in the defeated group ($p<.01$, unpaired t-test) (Figures 7C and 7D). This effect is attributable to differences in IP_3R sensitivity between groups and not to an inability to induce LTP in cells from control animals, as robust LTP could be induced in the control group with a higher concentration of IP_3 (500 μJ•μM) (Figure 7E).

Repeated stress alters NMDAR expression in certain brain regions, although the direction of change varies by region (Costa-Nunes et al., 2014; Fitzgerald et al., 1996; Yuen et al., 2012). To test for changes in global NMDAR expression, 10 μM NMDA was applied by bath perfusion and the change in

holding current was measured (Figure 8A). The peak current induced by 1-min NMDA perfusion did not differ between defeated animals and controls, indicating that repeated social stress does not affect total NMDAR expression (Figure 8B).

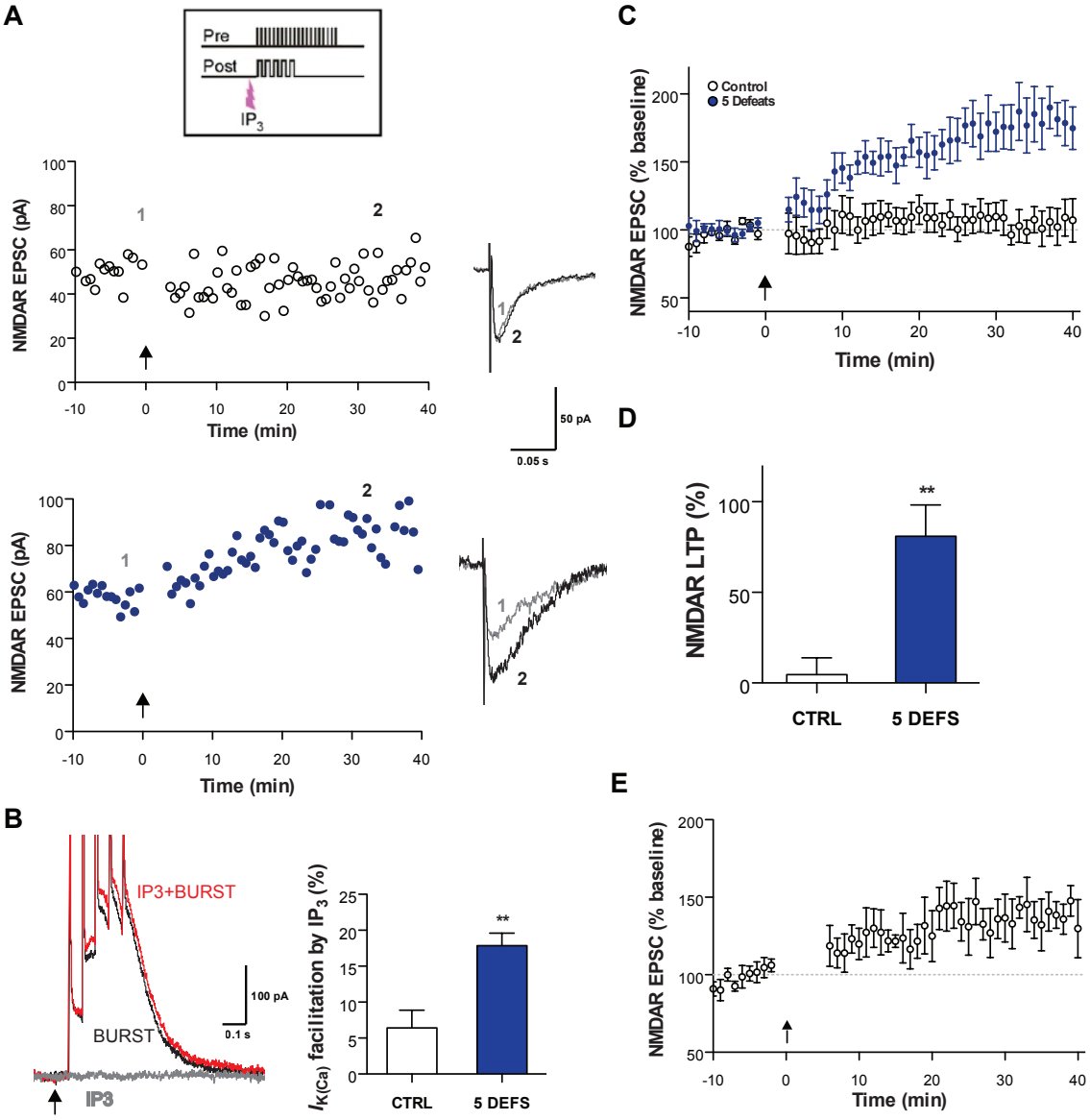
A
Pre
Post
IP3
NMDAR EPSC (pA)
Time (min)
50 pA
0.05 s
B
IP3+BURST
BURST
IP3
100 pA
0.1 s
IK(Ca) facilitation by IP3 (%)
CTRL
5 DEFS
C
Control
5 Defeats
NMDAR EPSC (% baseline)
Time (min)
D
NMDAR LTP (%)
CTRL
5 DEFS
E
NMDAR EPSC (% baseline)
Time (min)

Figure 7. NMDAR-mediated transmission is more susceptible to LTP induction after social defeat.

(**A**) Schematic of LTP induction protocol (top), example experiments to induce NMDAR-LTP in VTA DA neurons from control (middle) and defeated (bottom) rats. Time graph of NMDAR EPSCs (left) and example traces (right) from baseline (black) and post-induction (gray) time points indicated on the time graph. The LTP induction protocol was applied at t=0. (**B**) Example trace (left) and summary (right) of $I_{K(Ca)}$ facilitation by IP_3 prior to the LTP induction protocol. (t_{12}=3.81, $^{**}p$<.001, Student's unpaired *t*-test). (**C**) Summary time graph of baseline-normalized NMDAR-EPSCs. (control= 7 from 7 rats; 5 defeats= 7 from 7 rats). (**D**) Summary of NMDAR-LTP magnitude in control and defeated rats (t_{12}=3.93, $^{**}p$<.01, Student's unpaired *t*-test). (**E**) Summary time graph of baseline-normalized NMDAR-EPSCs. In this experiment, robust LTP was induced in VTA DA neurons from control rats (n=4 from 4 rats) using a higher concentration of IP_3 (500μJ•μM) than in (**B**) (250μJ•μM).

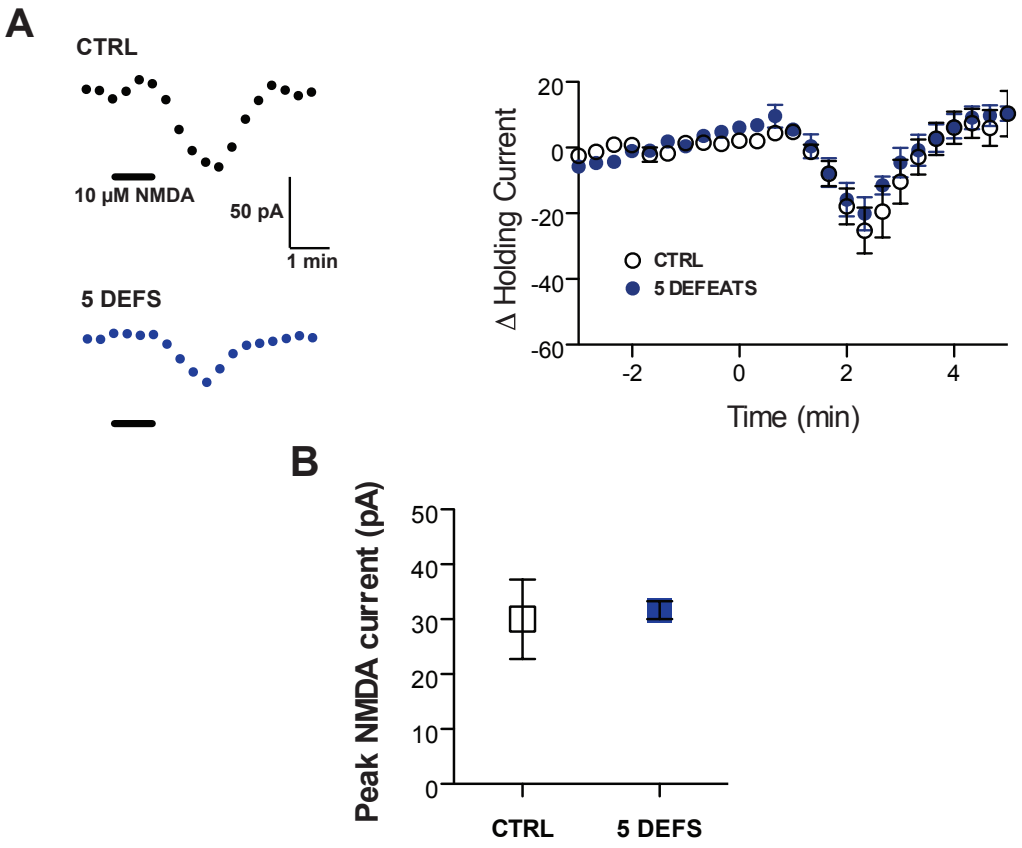

Figure 8: Global NMDA currents are unaltered by social defeat.

(**A**) Example traces (left) and summary (right) of inward currents induced by 1-min perfusion of 10μM NMDA in VTA neurons from control and defeated rats. (**B**) Summary of peak NMDA-induced current (control= 8 from 3 rats; 5 defeats =6 from 2 rats; t_{12}=.19, p=.86; Student's unpaired *t*-test).

REPEATED SOCIAL STRESS DOES NOT ALTER *IN VITRO* TONIC FIRING

Stress can modulate tonic activity of VTA DA neurons, as both increased and decreased *in vivo* tonic firing activity have been reported after repeated stress (Cao et al., 2010; Krishnan et al., 2007; Tye et al., 2013). These conflicting findings may be due to differences in the duration or severity of the stress protocol. To assess the effects of the repeated social defeat paradigm in this study, loose-patch recordings were made of DA cell firing in slices (Figure 9A). Stress did not significantly alter the tonic firing rate in these conditions (Figure 9B).

GLUCOCORTICOID RECEPTORS ARE NECESSARY BUT NOT SUFFICIENT FOR STRESS-INDUCED IP_3R SENSITIZATION

A major consequence of stress-induced HPA axis activation is the secretion of glucocorticoid hormone into the bloodstream. In the rat, social defeat stimulates robust corticosterone release that does not habituate in repeated defeat paradigms (Covington and Miczek, 2005). The next experiment sought to determine whether stress-evoked corticosterone release is necessary for the observed increase in IP_3R sensitivity with repeated stress. Corticosterone activates both glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs), but MRs are typically saturated by circadian fluctuations in corticosterone, while lower-affinity GRs are activated by levels attained with stress (Joels and de

Kloet, 1994). Therefore, the role of GRs was examined by treating rats with the GR antagonist mifepristone (40 mg/kg) or vehicle 30 min prior to each social defeat session (Figure 10A). The effect of DHPG on burst $I_{K(Ca)}$ was significantly enhanced in rats that received 5 social defeats in the vehicle-treated group ($p<.05$, Bonferroni post hoc test), but there was no effect of stress in the mifepristone-treated group. Thus, blockade of GRs during the stressor prevented the development of IP_3R sensitization. To determine whether GR activation alone is sufficient to sensitize IP_3Rs, rats were treated with corticosterone (2.5, 5, or 15 mg/kg) for 5 days (Figure 10C). 2.5 mg/kg corticosterone produces a comparable blood concentration to that evoked by a moderate stressor (15 minute intermittent footshock (Graf et al., 2013)), while doses ranging from 10-25 mg/kg have been used to simulate levels evoked by severe stress (Akirav et al., 2004). None of the tested doses significantly altered the DHPG effect on burst $I_{K(Ca)}$. GR signaling is therefore necessary for IP_3R sensitization, but not sufficient.

GLUCOCORTICOID RECEPTORS ARE NOT NECESSARY FOR COCAINE-INDUCED IP_3R SENSITIZATION

A previous study found that repeated psychostimulant treatment sensitizes IP_3Rs via PKA-dependent mechanism and enhances NMDAR-LTP (Ahn et al., 2010). As psychostimulants stimulate corticosterone secretion (Mantsch et al., 2000; Sarnyai, 1998), GR signaling could also mediate psychostimulant-induced IP_3R sensitization. To test this possibility, rats were treated with mifepristone (40

mg/kg) or vehicle 30 minutes prior to injection of cocaine (10 mg/kg) or saline for 5 days. The effect of DHPG on burst $I_{K(Ca)}$ was significantly larger in cocaine-treated animals ($p<.05$, Bonferroni post-hoc test) regardless of vehicle or mifepristone pretreatment (Figure 10B). From these data, it can be concluded that GR signaling is not involved in psychostimulant-induced IP_3R sensitization, although both psychostimulants and stress ultimately converge upon the PKA pathway.

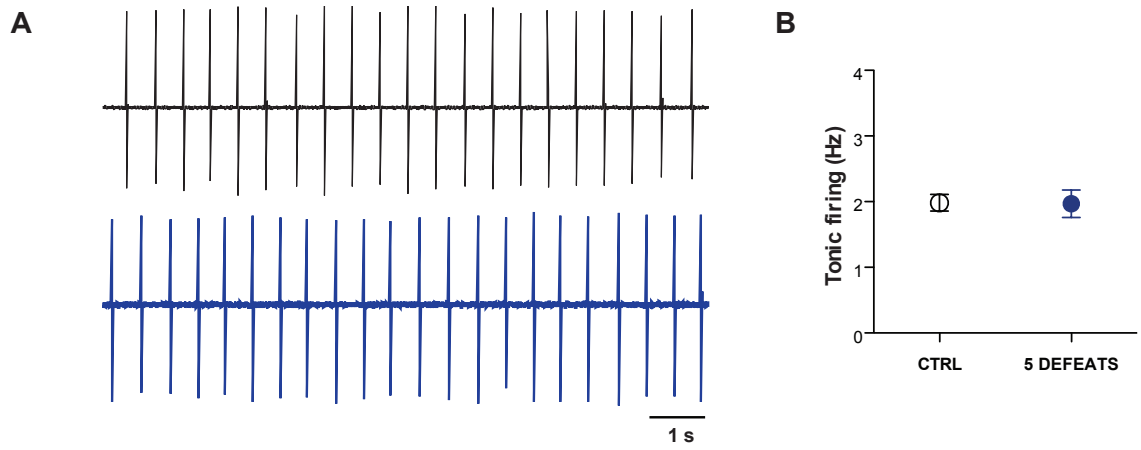

Figure 9: Tonic firing is unaltered by social defeat.

Example traces (**A**) and summary (**B**) of tonic firing frequency detected with loose-patch cell-attached recording in VTA neurons from control and defeated rats. (control = 15 from 3 rats; 5 defeats = 9 from 3 rats; t_{22}=.066, p=.95; Student's unpaired t-test)

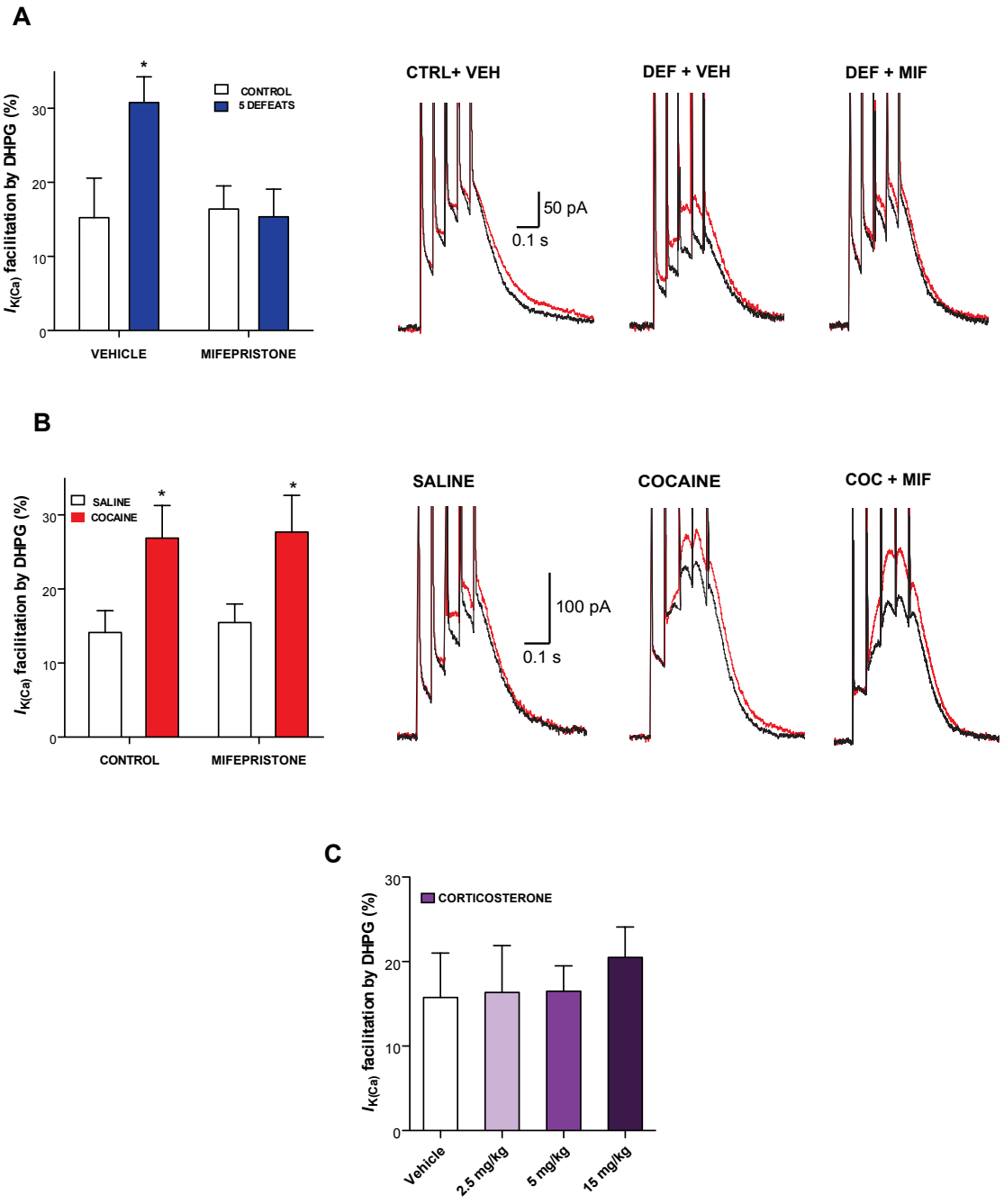
A
CONTROL
5 DEFEATS
$I_{K(Ca)}$ facilitation by DHPG (%)
VEHICLE
MIFEPRISTONE
CTRL+ VEH
DEF + VEH
DEF + MIF
50 pA
0.1 s
B
SALINE
COCAINE
CONTROL
MIFEPRISTONE
SALINE
COCAINE
COC + MIF
100 pA
0.1 s
C
CORTICOSTERONE
Vehicle
2.5 mg/kg
5 mg/kg
15 mg/kg

Figure 10. Stress-induced, but not cocaine-induced, IP_3R sensitization is sensitive to glucocorticoid receptor blockade.

(**A**) Summary (left) and example traces (right) of DHPG-induced burst $I_{K(Ca)}$ facilitation in VTA DA neurons from animals that were pretreated with mifepristone (40mg/kg, i.p.) or vehicle 30 min before undergoing each of 5 control or social defeat sessions. (vehicle + control= 8 from 5 rats; vehicle + 5 defeats = 10 from 4 rats; mifepristone + control= 10 from 5 rats; mifepristone + defeat = 11 from 4 rats. defeat: $F_{1,35}$=3.50, p=.07; mifepristone: $F_{1,35}$=3.39, p=.07; defeat x mifepristone: $F_{1,35}$=4.56, p<.05; 2-way ANOVA). *p<.05, Bonferroni post hoc test. (**B**) Summary (left) and example traces (right) of DHPG-induced burst $I_{K(Ca)}$ facilitation in VTA DA neurons from animals that were or were not pretreated with mifepristone (40mg/kg, i.p.) 30 min before undergoing each of 5 saline or cocaine (10 mg/kg, i.p.) injections. (control+ saline= 17 from 7 rats; control+ cocaine = 16 from 7 rats; mifepristone + saline = 21 from 8 rats; mifepristone + cocaine = 16 from 6 rats. cocaine: $F_{1,66}$=11.4, p<.01; mifepristone: $F_{1,66}$=.086, p=.77; cocaine x mifepristone: $F_{1,66}$=.005, p=.94; 2-way ANOVA). *p<.05, Bonferroni post hoc test. (**C**) Summary of DHPG-induced burst $I_{K(Ca)}$ facilitation in VTA DA neurons from animals that were treated with 5 daily vehicle or corticosterone (2.5, 5, or 15 mg/kg) i.p. injections. (vehicle = 8 from 6 rats; 2.5 mg/kg corticosterone = 11 from 5 rats; 5 mg/kg corticosterone = 10 from 4 rats; 15 mg/kg corticosterone = 12 from 7 rats. $F_{3,37}$=.260, p=.85, 1-way ANOVA).

REPEATED SOCIAL STRESS PROMOTES LEARNING OF DRUG-ASSOCIATED CONTEXTUAL CUES IN A GLUCOCORTICOID RECEPTOR-DEPENDENT MANNER

Manipulations that increase IP_3R sensitivity may enhance learning of drug reward-related cues. Therefore, the effect of social defeat stress was tested on acquisition of cocaine conditioned place preference (CPP), a Pavlovian task in which the animal learns to associate a particular environment with drug reward. Rats underwent stress or control procedures for 5 days, then were tested for initial chamber preference one day later. The next day (two days post-stress), rats were conditioned with a single injection of 5 mg/kg cocaine. Rats were then tested for post-conditioning chamber preference the following day. This brief conditioning paradigm was chosen to avoid cross-sensitization of IP_3Rs by repeated cocaine exposure, as a single psychostimulant treatment does not sensitize IP_3Rs (Ahn et al., 2010). Stressed rats displayed a large, significant preference for the cocaine-paired chamber after conditioning ($p<.001$, paired Student's *t*-test), while naïve and handled controls showed slight ($p<.05$) or no significant preference, respectively (Figure 11A). The CPP score, calculated as the change in percent preference with conditioning, was significantly larger in stressed rats compared to naïve and handled controls ($p<.001$, Bonferroni post hoc test) (Figure 11B). These data demonstrate that learning of cocaine-associated cues is enhanced following repeated social defeat.

It was next asked whether glucocorticoid receptor signaling, which is necessary for stress-enhancement of IP_3R sensitivity, also plays a role in promoting acquisition of cocaine CPP. As in the electrophysiological experiments, rats were treated with 40 mg/kg mifepristone or vehicle 30 minutes before each social defeat session. An additional group received mifepristone followed by the handled control procedure. While vehicle-treated stressed rats acquired significant place preference ($p<.001$, paired Student's *t*-test), mifepristone-treated stressed or control rats did not acquire significant preference (Figure 11C). Mifepristone-treated stressed rats displayed significantly smaller CPP scores than vehicle-treated stressed rats ($p<.05$, Bonferroni post hoc test), as did mifepristone-treated controls ($p<.05$, Bonferroni post hoc test)(Fig 11D). These results indicate that glucocorticoid receptor activation during stress is required to enhance subsequent acquisition of CPP.

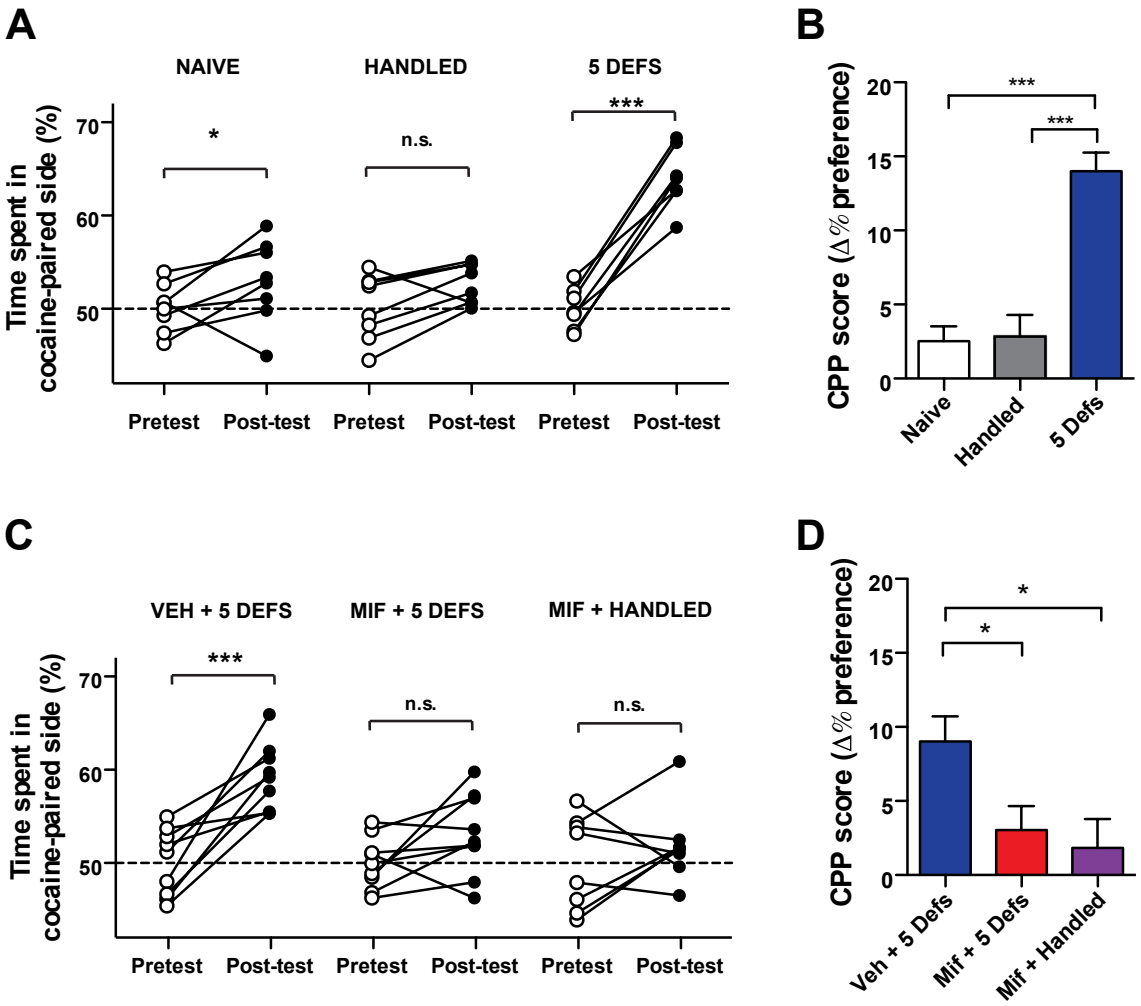

Figure 11. Social defeat promotes cocaine-induced CPP via a glucocorticoid receptor-dependent mechanism.

(A) Summary of changes in the preference for the cocaine-paired side of the CPP apparatus following 1-day conditioning with cocaine (5 mg/kg, i.p.) in naïve, handled, and defeated rats. (naïve = 8; handled = 8; 5 defeats = 7; naïve: t_7=2.51, *p<.05; handled: t_7=1.90, p=.10; 5 defeats: t_6=11.0, ***p<.001, Student's paired t-test). **(B)** Summary of 1-day cocaine CPP scores in naïve, handled, and defeated rats ($F_{2,20}$= 25.2 , p<.0001, 1-way ANOVA). ***p<.001, Bonferroni post hoc test. **(C)** Summary of changes in the preference for the cocaine-paired side of the CPP apparatus following 1-day conditioning with cocaine (5 mg/kg, i.p.) in rats pretreated with vehicle or mifepristone (40 mg/kg, i.p.) 30 min prior to social defeat or handling sessions (vehicle + 5 defeats = 9; mifepristone + 5 defeats = 9; mifepristone + handled = 8. vehicle + 5 defeats: t_8=5.30, ***p<.001; mifepristone + 5 defeats: t_8=1.90, p=.09; mifepristone + handled: t_7=.95, p=.37; Student's paired t-test). **(D)** Summary of 1-day cocaine CPP scores in rats treated with vehicle+5 defeats, mifepristone + 5 defeats, and mifepristone + handling ($F_{2,23}$= 4.90, p<.05, 1-way ANOVA). *p<.05, Bonferroni post hoc test.

Chapter 4: Discussion

Learning of drug-associated cues is enhanced following periods of stress. Stress has been reported to induce metaplasticity, i.e. experience-dependent changes in the threshold for synaptic plasticity induction and/or the acquisition of learned behaviors, in certain brain regions (Abraham, 2008; Bains et al., 2015; Kim and Yoon, 1998; Schmidt et al., 2013). Metaplastic changes following stress typically impede induction of LTP and enhance that of LTD (Kim and Yoon, 1998; Schmidt et al., 2013). In the present study, it was found that repeated social defeat enhanced induction of LTP of NMDAR transmission in DAergic neurons of the VTA, a midbrain dopaminergic nucleus that is critically involved in reward-based learning and addiction. This social defeat paradigm also promoted acquisition of cocaine conditioned place preference, a form of Pavlovian reward learning that requires NMDAR-mediated bursting in DA neurons (Zweifel et al., 2008; Zweifel et al., 2009).

REPEATED STRESS SENSITIZES IP_3RS IN VTA DA NEURONS

The data show that repeated social defeat enhanced sensitivity of IP_3Rs, which serve as coincidence detectors of presynaptic activity and neuronal bursting in VTA DA neurons and are part of the NMDAR-LTP induction pathway

(Harnett et al., 2009). Phosphorylation of IP_3Rs by PKA is necessary to maintain the enhanced sensitivity after defeat, as inhibition of PKA activity reversed the effect. Stress, including social defeat, evokes bursting in DA neurons (Anstrom et al., 2009; Anstrom and Woodward, 2005; Brischoux et al., 2009) and induces DA efflux in the NAc (Boyson et al., 2014; Holly et al., 2015; Kalivas and Duffy, 1995). Bursting also releases DA locally from somata and dendrites (Deutch et al., 1985; Geffen et al., 1976), activating somatodendritic D2 receptors (Beckstead et al., 2004). Chronic stimulation of G_i-coupled receptors, such as D2Rs, is known to upregulate the cAMP-PKA pathway (Hyman et al., 2006; Nestler and Aghajanian, 1997; Nevo et al., 1998). Repetitive stress-induced somatodendritic dopamine release may sensitize IP_3Rs via this mechanism. Both adenylate cyclase and PKA protein subunits have been reported to increase with repeated G_i stimulation. There was no difference among groups in hyperpolarization-evoked I_h currents, which are sensitive to cAMP levels but are insensitive to PKA phosphorylation; therefore enhanced IP_3R phosphorylation is likely achieved by PKA upregulation.

GLUCOCORTICOID RECEPTORS ARE NECESSARY, BUT NOT SUFFICIENT, FOR STRESS-INDUCED IP_3R SENSITIZATION

Glucocorticoid receptor signaling during the defeat sessions is necessary for stress-mediated enhancement of IP_3R sensitivity, as treatment with the GR

antagonist mifepristone prior to each social defeat session prevented sensitization of IP_3Rs. Stress-induced DA release in the NAc is regulated by glucocorticoids (Rougé-Pont et al., 1998), but the mechanism of regulation is not fully understood. Glucocorticoids may act directly on DA neurons, as approximately 60% express GRs (Harfstrand et al., 1986; Hensleigh and Pritchard, 2013), and application of corticosterone has been reported to potentiate NMDA responses and enhance glutamate-induced bursting in DA neurons both *in vitro* and *in vivo* (Cho and Little, 1999; Overton et al., 1996). However, selective deletion of GRs in D1-expressing accumbens medium spiny neurons severely attenuates social defeat-induced DA release in the NAc (Barik et al., 2013), therefore corticosterone may predominantly shape DA release by modulating MSN-mediated disinhibition of VTA DA neurons (Xia et al., 2011). A recent study also demonstrated that social defeat-induced DA release into the NAc shell requires CRF-R2 signaling in the VTA during stress (Holly et al., 2015), which may be a consequence of short-term VTA NMDAR potentiation by CRF-R2 (Ungless et al., 2003). CRF-R1 signaling also appears to support defeat-induced DA release (Boyson et al., 2014). CRF synthesis and activation of CRF-releasing neurons are positively modulated by glucocorticoids in the central nucleus of the amygdala and the BNST, both major sources of CRF input to the VTA (Kolber et al., 2008; Makino et al., 1994a, b; Watts, 1996; Watts and Sanchez-Watts, 1995). GR blockade may therefore attenuate stress-induced DA release by dampening CRF input to the VTA. The mechanisms outlined above

are not mutually exclusive and likely act in concert to regulate stress-evoked DA release.

Although stress-induced IP_3R sensitization is sensitive to GR blockade, repeated administration of corticosterone did not reproduce the effect of stress at any of the doses tested. Together, these data indicate that GR signaling is necessary but not sufficient for IP_3R sensitization. If repeated D2 activation by stress-induced DA release underlies IP_3R sensitization, these results are not surprising, as the glutamatergic input that drives bursting is inactive in unstressed animals and therefore cannot be amplified by GR signaling. Figure 12 summarizes putative sites of GR action that may mediate its permissive effect on metaplasticity.

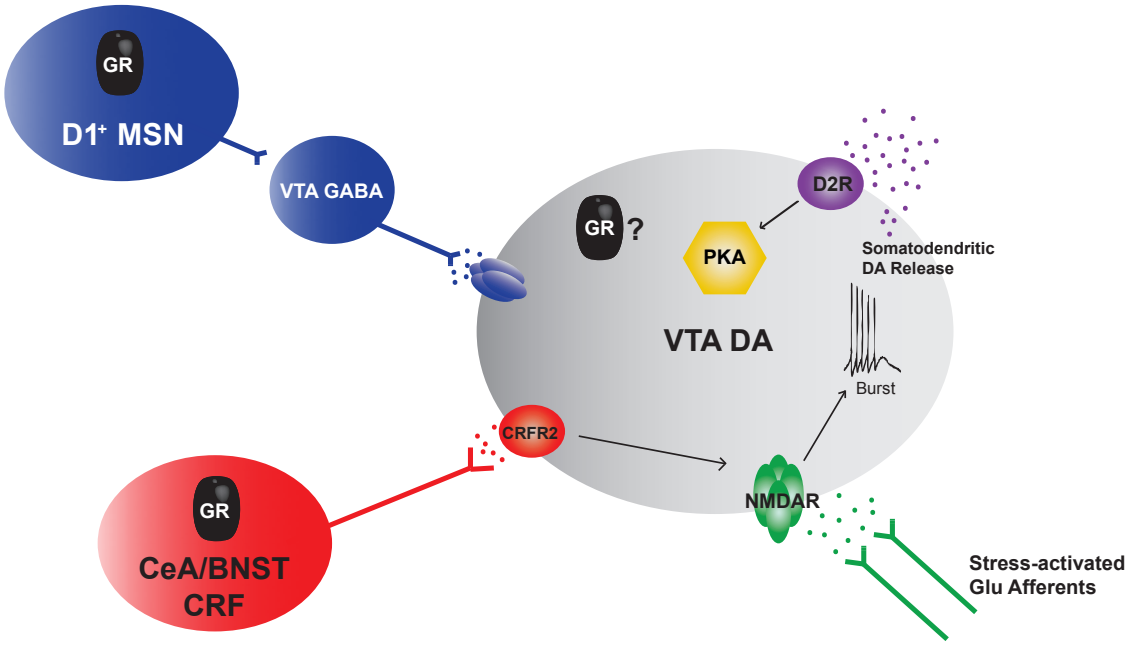

Figure 12. Hypothetical pathway for stress-induced metaplasticity.

Simplified diagram depicting a potential pathway for the induction of metaplasticity by stress in VTA DA neurons. Here, glutamatergic afferents activated by stress induce NMDAR-dependent bursting, which causes somatodendritic release of DA. Repeated stimulation of D2 receptors upregulates PKA to express NMDAR metaplasticity. Bursting is increased by (1) transient CRFR2-dependent upregulation of NMDARs, which may be regulated by GRs in CRFergic neurons, and (2) GR-mediated disinhibition of DA neurons by D1-expressing NAc medium spiny neurons. It is unknown if GRs in VTA DA neurons are involved. Neurotransmitters are labeled as follows: green, glutamate; purple, dopamine; blue, GABA; red, CRF.

GLUCOCORTICOID RECEPTORS ARE UNNECESSARY FOR COCAINE-INDUCED IP_3R SENSITIZATION

Previous studies found that repeated administration of ethanol (Bernier et al., 2011) and amphetamine (Ahn et al., 2010), both of which elicit DA release (Kohl et al., 1998; Mercuri et al., 1989; Yan et al., 1996), also induce PKA-dependent IP_3R sensitization. Drugs of abuse stimulate corticosterone secretion (Armario, 2010), therefore the role of GRs in psychostimulant-induced IP_3R sensitization was examined. Consistent with previous findings using amphetamine, repeated cocaine treatment resulted in IP_3R sensitization; however, GR signaling was unnecessary for this effect. Under GR blockade, levels of DA released into the VTA by cocaine alone are likely sufficient to induce D2-mediated changes in the PKA pathway, whereas stress cannot appreciably elevate DA levels without intact GR signaling.

REPEATED STRESS ENHANCES NMDAR LTP

The magnitude of NMDAR LTP induction was significantly larger in neurons from socially defeated animals 24-48 hours after stress. LTP was induced by uncaging a fixed concentration of IP_3 just prior to a burst of APs paired with brief synaptic stimulation. Because the concentration of IP_3 during LTP induction was controlled across cells, it can be concluded that the larger

LTP magnitude in defeated animals is due to enhanced IP_3R sensitivity. There is evidence for GC-mediated potentiation of L-type Ca^{2+} channel currents in the hippocampus and amygdala (Chameau et al., 2007; Karst et al., 2002), and L-type Ca^{2+} channels are necessary for NMDAR LTP induction, as the LTCC antagonist isradipine blocks LTP (Degoulet et al., 2015). However, pharmacological agonists of LTCCs do not increase NMDAR LTP magnitude (Degoulet et al., 2015), therefore stress-induced changes in LTCCs are unlikely to impact the results. Because CRF, which is released into the VTA during stress, has been shown to transiently potentiate NMDAR transmission (Ungless et al., 2003), global NMDAR function was also assessed. There was no difference in global NMDA transmission at this time point, consistent with rapid reversal of CRF-mediated NMDAR potentiation.

REPEATED STRESS DOES NOT ALTER TONIC FIRING

Studies have yielded conflicting results regarding the effect of stress on DA neuronal firing patterns. 10 days of social defeat increased tonic firing and bursting in a subset of animals (Cao et al., 2010; Chaudhury et al., 2013), 2 weeks of cold room exposure left tonic and burst firing unaltered (Valenti et al., 2012), and 8-12 weeks of chronic variable stress increased tonic and burst firing. In the present study, 5 days of social defeat did not alter the tonic firing rate measured in slices, nor were changes observed in I_H, which is implicated in

stress-mediated increases in firing frequency (Cao et al., 2010). Stress effects frequently take on U-shaped response curves, with moderate/brief stressors and prolonged/severe stressors yielding opposite results (Sapolsky, 2015). The conflicting findings described above may represent such a U-shaped curve if both stressor severity and duration of exposure are taken into account. Alternatively, different stressor types (e.g., psychological vs. physical, or painful vs. painless aversive) may simply exert different effects on DA neuronal activity. Systematic experiments varying the duration of fixed stressor types and comparisons across stressor types are necessary to clarify the relationship of stress to DA activity.

REPEATED STRESS ENHANCES LEARNING OF DRUG-ASSOCIATED CUES

The data show that repeated social defeat enhances learning of reward-associated cues, assessed with a conditioned place preference paradigm. NMDAR LTP may contribute to this form of associative learning, as prior studies showed that CPP acquisition is sensitive to pharmacological antagonists of components of the NMDAR-LTP pathway. Intra-VTA blockade of NMDARs, group I mGluRs, PKA, or L-type calcium channels impair both CPP and NMDAR LTP induction (Ahn et al., 2010; Degoulet et al., 2015; Harnett et al., 2009; Whitaker et al., 2013). In this study, defeated animals acquired CPP following a single training session with a modest dose of cocaine that was insufficient to

produce learning in controls, and the effect of defeat was blocked by pretreatment with mifepristone before the stressor. These data are consistent with other reports of augmented Pavlovian reward learning following stressful experience (Burke et al., 2011; Der-Avakian et al., 2007; Kreibich et al., 2009). (but see (Papp et al., 1991)).

Facilitated reward learning after stress is frequently ascribed to enhanced drug reward, as prior stress increases drug-induced DA release and locomotor activation in response to a psychostimulant challenge, a phenomenon known as cross-sensitization (Boyson et al., 2014; Garcia-Keller et al.; Han et al., 2015; Lepsch et al., 2005; Pacchioni et al., 2006; Yap and Miczek, 2007). From a prediction error perspective, larger drug rewards yield larger positive prediction errors, resulting in enhanced learning. In the NMDA LTP scheme, enhanced reward would be equivalent to more pairings (i.e., more frequent bursting in the presence of the cue). However, as stress cross-sensitization has only been demonstrated with psychostimulants and opioids (Rouge-Pont et al., 1995), it unknown whether enhanced drug reward can explain enhancing effects of stress on addiction-like behaviors with other classes of abused drugs. This study provides evidence that stress facilitates a synaptic plasticity mechanism that may contribute to learning of drug-associated cues, thereby expediting acquisition of conditioned place preference independently of changes in drug reward. However, as cocaine CPP was used to test reward learning in this study, NMDAR metaplasticity cannot be distinguished from enhanced drug reward. Stress cross-

sensitization to drugs of abuse is also dependent upon GR signaling, so the mifepristone experiment cannot provide insight into this confound. IP_3R sensitization should also enhance learning driven by non-drug rewards, but there is evidence that stress, via ghrelin signaling, enhances the rewarding effect of food as well (Chuang et al., 2011). It may be possible to resolve the relative contribution of IP_3R sensitization by treating animals with intra-VTA H89, a PKA inhibitor, before conditioning. Like PKI, H89 should normalize PKA-dependent IP_3R phosphorylation between groups, eliminating the contribution of IP_3R sensitization to behavior. In a previous publication, H89 was shown to attenuate, but not abolish, amphetamine CPP induced with 3 days of conditioning (Ahn et al., 2010).

FUTURE DIRECTIONS

Social stress, psychostimulants, ethanol, and social isolation have been shown to induce NMDAR metaplasticity. So far, the working hypothesis for the molecular mechanism of this effect is that repeated drug or stress-evoked DA release acts via D2 receptors to upregulate PKA function. This mechanism could be tested by administering intra-VTA D2 antagonists prior to each stress session or drug treatment. Furthermore, the role of NMDAR-mediated bursting in the induction of IP_3R sensitization has not been examined. Recent work has suggested that generation of phasic bursting is a common mechanism of all

drugs of abuse (Covey et al., 2014), and stress triggers bursting as well (Anstrom et al., 2009). The role of NMDARs could be examined by administering intra-VTA AP5 prior to each stress or drug treatment. Determining the necessity of bursting would provide mechanistic insights into the likely site(s) of GR action. In the hypothetical mechanism described in Figure 12, GRs act to amplify bursting driven by aversive cue input in two ways: (1) GRs on CRFergic neurons increasing CRF input to VTA DA neurons, transiently enhancing NMDAR function and (2) GRs on MSNs disinhibit VTA neurons. The relative importance of these GR sites could be tested by local mifepristone infusion to the VTA, BNST, amygdala, and NAc. A role for corticosterone in 'amplifying' an existing phasic aversion signal is attractive because the slow kinetics of corticosterone release would selectively induce IP_3R sensitization when aversive experiences are ongoing, thus filtering minor pains or startles that activate DA neurons but do not constitute true 'stress.'

Studies from our lab, including this dissertation, have exclusively used the conditioned place preference paradigm to assess the behavioral significance of manipulations that induce NMDAR metaplasticity *in vitro*. However, there is some evidence to suggest that DA neurons also function in conditioned avoidance and escape behavior (Fenu and Di Chiara, 2003; Oleson and Cheer, 2013; Tye et al., 2013). It may be worthwhile to examine the effect of NMDA metaplasticity on these non-reward behaviors. For example, certain animals exhibit social avoidance after repeated social defeat (Berton et al., 2006). Social avoidance

has been used as an index of depression in some studies; however an alternate interpretation of this finding is learned avoidance of novel animals based on the history of social defeat, as other chronic stress models produce enhanced social aggression rather than avoidance (Sandi and Haller, 2015).

CONCLUSIONS

The main finding of this dissertation is that stress acts as a metaplastic signal, facilitating induction of NMDAR-LTP at VTA synapses by increasing IP_3R sensitivity to IP_3 after the cessation of stressful experience. Metaplastic effects of stress have been observed in several brain regions, first in hippocampal CA1 synapses, where stress impairs LTP induction (Diamond et al., 1996; Foy et al., 1987; Shors et al., 1989) while enhancing LTD induction (Xu et al., 1997). Similar to the present findings in VTA DA neurons, metaplastic effects on both hippocampal LTP and LTD require GR signaling during the stressor (Kim et al., 1996; Xu et al., 1998; Yang et al., 2004, 2005). LTP is similarly impaired in the medial prefrontal cortex following stress (Maroun and Richter-Levin, 2003; Rocher et al., 2004), and corticosterone treatment mimics the effects of stress (Cerqueira et al., 2005). By contrast, stress enhances subsequent LTP induction via metaplasticity in the lateral amygdala (Sarabdjitsingh et al., 2012) (Suvrathan et al., 2013), a region in which synaptic plasticity is critical to acquire Pavlovian conditioned fear responses. Taken together, these findings suggest that stress, typically via GR signaling, induces a set of metaplastic effects which bias the

brain towards utilizing Pavlovian mechanisms at the expense of other forms of learning. The present study has described glucocorticoid-dependent sensitization of a synaptic plasticity mechanism that is accompanied by enhanced Pavlovian reward learning following stress. These findings may illuminate one mechanism by which stress increases vulnerability to addiction, a chronic, relapsing disorder that is perpetuated by powerful memories of stimuli associated with drug reward.

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